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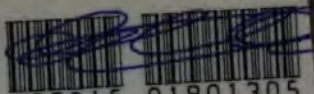
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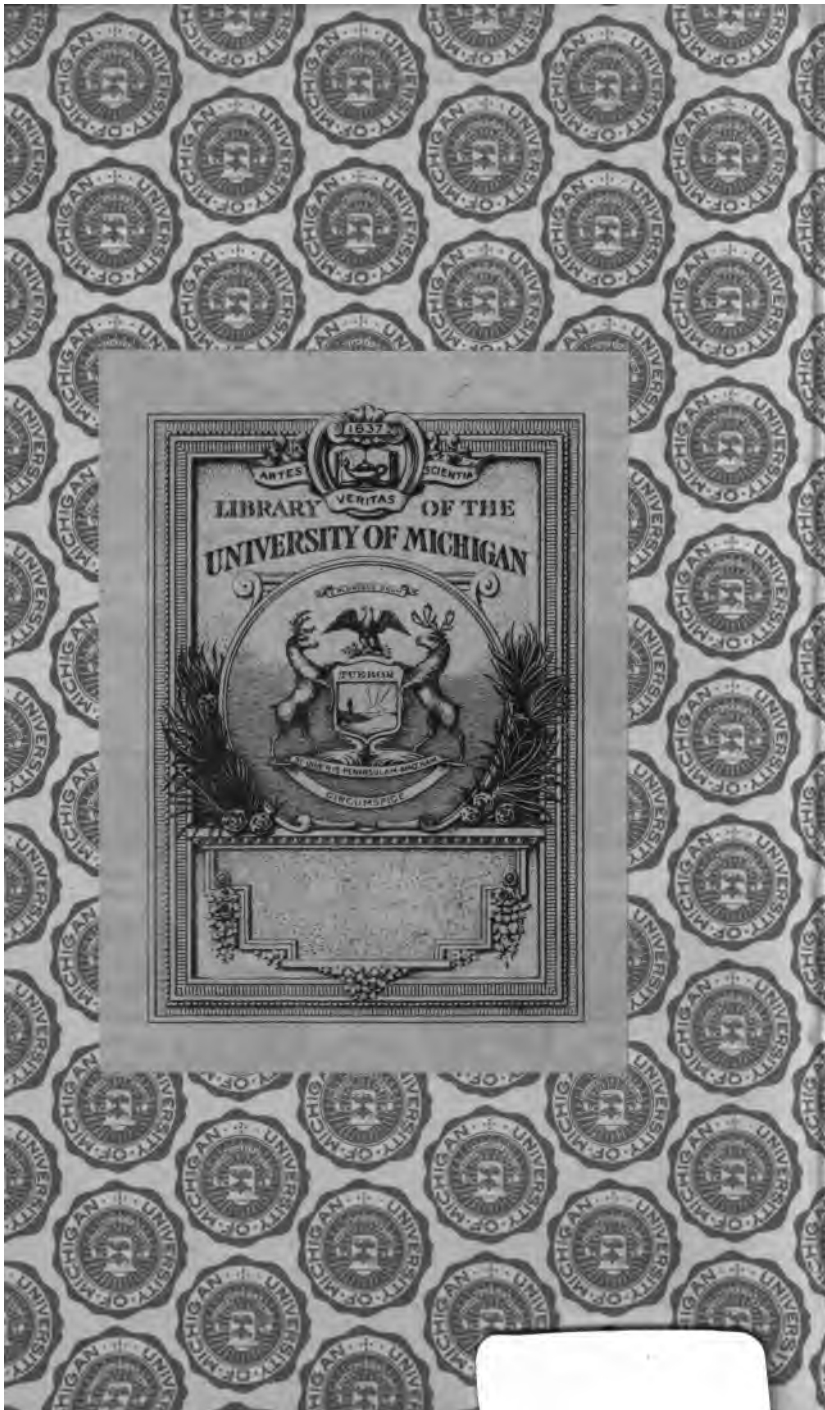
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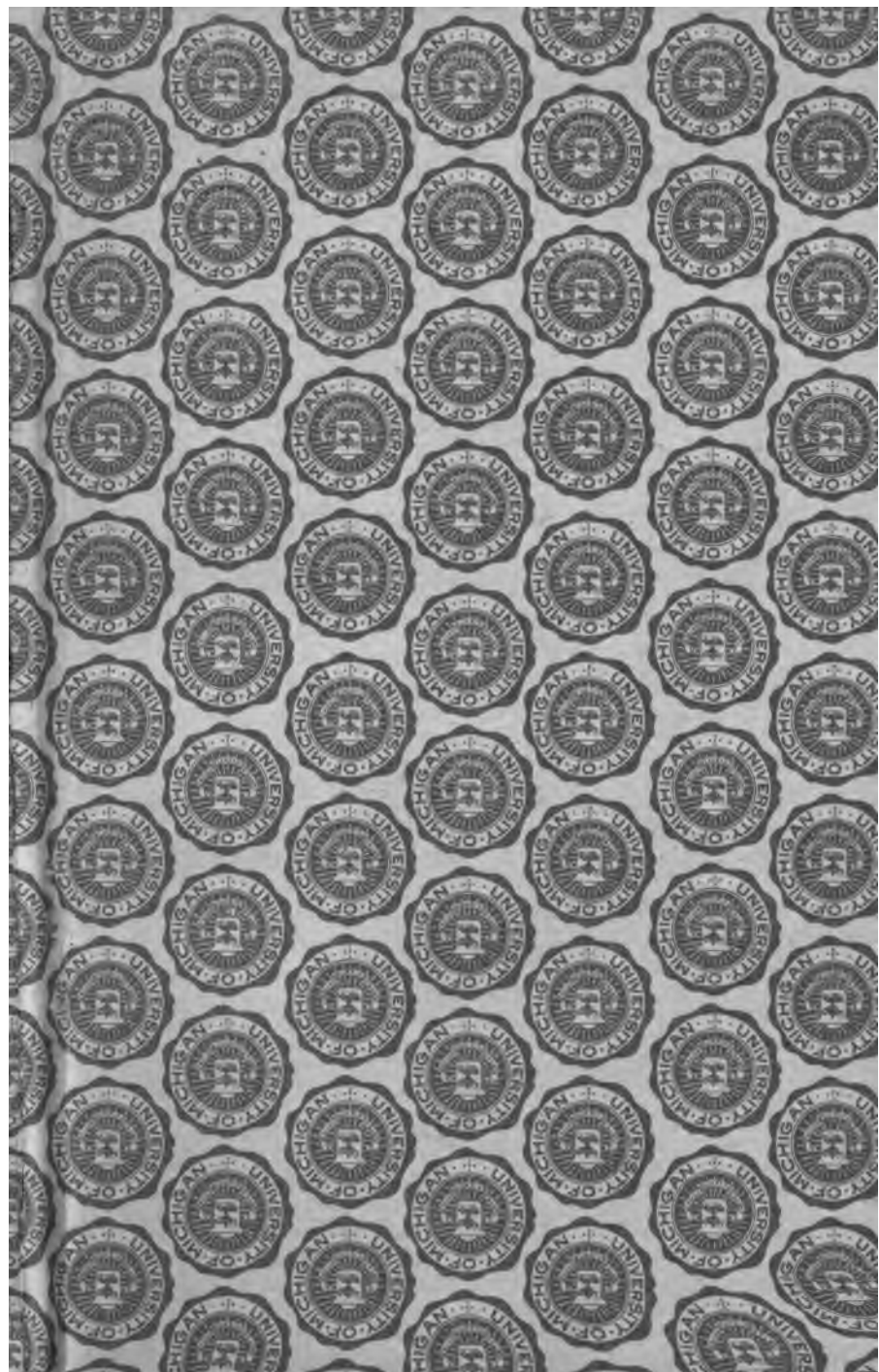
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LABORATORY MANUAL
IN
GENERAL MICROBIOLOGY

PREPARED BY THE
LABORATORY OF BACTERIOLOGY AND HYGIENE
MICHIGAN AGRICULTURAL COLLEGE

FIRST EDITION
SECOND THOUSAND

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PREFATORY NOTE

LABORATORY instruction in bacteriology at the Michigan Agricultural College developed under the direction of Dr. C. E. Marshall. This laboratory guide represents the accumulated efforts of instructors working for a period in excess of a decade. To Assistant Professor L. Zae Northrup is due the credit for collecting and arranging the material presented as well as for preparing *de novo* many of the experiments and much of the supplementary matter. She has been assisted by Mr. W. L. Kulp. Dr. E. T. Hallman and Dr. L. R. Himmelberger have taken the responsibility for arranging the exercises relating to immunity, serum therapy and pathologic bacteriology. Great praise is due Dr. F. H. H. Van Suchtelen for introducing many new features into the laboratory work during the academic year 1912-13, and also Dr. Otto Rahn for his several years of admirable effort immediately preceding. Others whose influence has been felt in creating this guide and to whom credit is due are Professors W. G. Sackett, S. F. Edwards, L. D. Bushnell, C. W. Brown and W. H. Wright.

While some claim to originality may be made for this laboratory guide, it is to be expected that much of the material herein has been presented in various other manuals and perhaps in better form in many instances. The greatest effort has been made to make this a laboratory guide to General Microbiology, leaving the particular fields of dairy, soil, water, medical and other phases of bacteriology to special guides already in print or at present projected. The presentation of this manual to the public is in no way an

intimation that the special fields have not been admirably dealt with by others.

The subject matter given under Part I of this manual is primarily for the purpose of giving a working knowledge of laboratory methods used in the study of microorganisms. Molds, yeasts and bacteria are taken up in the order of their comparative sizes and studied as to their identification by morphological and cultural methods. It is presupposed that the student has a knowledge of these microorganisms acquired from preceding lectures in microbiology.

Part II consists of exercises demonstrating the various physiological activities of microorganisms.

Part III deals with applied microbiology. After the student has familiarized himself with the ordinary tools and technic, etc., as dealt with in Parts I and II, it is not necessary that he be burdened with minute, detailed instructions. We have had this in mind in preparing Part III.

No attempt has been made to compile an exhaustive list of exercises; the aim has been only to cover a wide range of activities under each different subject. In many cases, exercises have been taken directly, with few or no modifications, from laboratory manuals already in print. Credit has not been given directly; the list of references, however, includes all books from which material has been taken.

The purpose of this laboratory manual is to make the student more independent. Practically all directions for work to be done are contained in it; for this reason the work as assigned from day to day should be read over carefully *before beginning an exercise* and then followed step by step. Any desirable changes in directions may be indicated by the instructor.

WARD GILTNER,
Head of Department.

EAST LANSING, MICH.
Sept. 1, 1915.

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APPENDIX

Outline for the Study of Microbiology—Special Media—Table for Identification of Bacteria in Polluted Water—Characters of B. Coli—B. Typhosus Groups—Common Disinfectants—So- lutions for Cleaning Glassware—Standard Solutions—Indi- cators—Salt Solutions—Test Solutions—Mounting Media— Stains—Solutions for Use in Staining—Steam Table—Tem- perature Conversion Formulæ—Metric System—List of Text and Reference Books.	331
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LABORATORY RULES

1. Do not bring coats, sweaters, hats, etc., into the laboratory and lay them on desks, etc.; hang them in the place provided for the purpose.

2. Before beginning and after finishing work, the top of the desks must be washed off with a liberal supply of 1-1000 mercuric chloride. This will destroy all micro-organisms and their spores and aid greatly in rendering aseptic technic possible. A large bottle of this disinfectant will be found near each desk.

3. Do not put string, paper, pencils, pins, etc., in the mouth nor moisten labels with the tongue while in the laboratory. Follow this practice outside of the laboratory also. Food should not be eaten in the laboratory.

4. Observe all possible cleanliness and neatness in the care of apparatus, desk, microscope, etc.

5. Apparatus must be kept inside the desks, but not cultures. Cultures must be kept at a constant temperature in the place fitted for this purpose.

The microscope and accessories must be returned to the case at the close of the work.

6. Water, gas, steam and electricity are to be turned off when not in use. This applies to the individual desks, large sinks, steam (including autoclav), hot-air sterilizers, etc.

7. Put all solid waste material, cotton, paper, matches, coagulated milk, etc., and waste liquid *which will solidify when cold* (agar, gelatin) into receptacles provided for that purpose, *not into the sinks*.

8. Apparatus, media, etc., should be removed from steam heaters, immediately after steaming.

9. No cultures are to be taken out of the laboratory without the permission of the head of the department.

10. All accidents, such as spilling infected material (pathogenic or non-pathogenic), cutting or pricking the fingers, must be reported at once to the instructor in charge.

Additional rules will be given if necessary, in conjunction with special exercises or technic.

11. 1-1000 mercuric chloride will not injure the skin if not used too often. Wash your hands in it thoroughly each time before you leave the laboratory to avoid carrying away undesirable organisms. **Use every precaution against infection.**

12. At the beginning of each laboratory period read over carefully the directions for the next exercise in order to understand its purpose and to make any necessary preliminary preparations.

13. Take careful notes on all observations made in the study of cultures and preparations made from them.

FORM FOR WRITING UP EXERCISES IN THE NOTEBOOK

I. Object. A concise statement of what the exercise is intended to prove or demonstrate is to be given.

II. Apparatus. This includes everything with the exception of the every-day tools such as burner, platinum needles, etc.

III. Cultures. A brief morphological and cultural description characteristic of each organism should be given, also its occurrence and importance. Certain organisms are used for a certain purpose. If this purpose is not evident, ascertain from the references given why these particular organisms were used.

IV. Method. State briefly but clearly your method of procedure.

V. Results. Give your results in full. Tabulate data so that they may be comprehended at a glance. Results often may be tabulated as + and -. Plot curves whenever possible.

VI. Conclusions. Draw the conclusion which *your own* results warrant.

VII. Error. You may know that your results and the consequent conclusions are in error. If so, state what you consider to be the correct results and conclusions, noting any irregularities or abnormalities which may have occurred to change the results.

VIII. Practical Application. Apply the principles involved in the exercise to some practical purpose.

IX. References. Give the substance of the references placed at the end of each exercise in your own words and apply to the exercise in question. Do not copy verbatim.

Note. In writing up the notebook, details under II and IV should be omitted, only the headings are necessary.

PART I

GENERAL MORPHOLOGICAL AND CULTURAL METHODS

EXERCISE 1. CLEANING GLASSWARE

Glassware for use in microbiological laboratory work should be not merely clean, but *chemically* clean. Test tubes, Petri dishes, flasks, etc., are the receptacles used in the microbiological laboratory for containing the different nutrient substances upon which microorganisms are to subsist. Very frequently free alkali may be present on new glassware in sufficient quantity to prevent microbial growths in the nutrients contained therein. Prescott and Winslow in testing out different glassware say that, "The more soluble glassware yielded sufficient alkali to the medium to inhibit four-fifths of the bacteria present in certain cases."

Glassware which *looks* clean may have been used previously and should be given a thorough cleaning to rid it of possible traces of mercuric chloride, or other chemical having germicidal properties.

Follow directions carefully and clean all new and apparently clean glassware in the order given.

Cleaning New or Apparently Clean Glassware. All new glassware should first be treated with chromic acid cleaning solution* (*see appendix for all formulæ*) before proceeding with the directions for cleaning glassware.

Return used cleaning solution to the glass receptacle provided for the purpose. Do not throw it away. This

* Chromic acid cleaning solution will be designated hereafter merely as cleaning solution.

solution may be used until oxidized, i.e., until dark green in color.

Heat will facilitate the action of the cleaning solution.

Small amounts of organic matter adhering to glassware are oxidized by this solution, but will not disappear until removed by a suitable brush and cleaning powder.*

New Petri dishes and test tubes may conveniently be

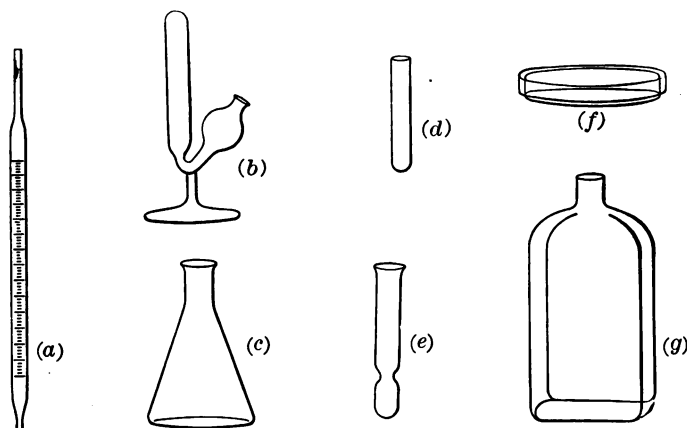


FIG. 1.—(a) Pipette, (b) Smith's Fermentation Tube, (c) Erlenmeyer Flask, (d) Test Tube, (e) Roux Tube, (f) Petri Dish, (g) Roux Flask.

placed in a large glass jar, covered with cleaning solution and allowed to stand over night. Heavy glass jars will not stand heating in steam. New flasks may be partially filled with cleaning solution and placed in steam for fifteen minutes.

Test Tubes. New test tubes should be filled with cleaning solution, placed in a wire basket and heated for

* Any inexpensive fine-grained cleaning powder as powdered pumice stone, Bon Ami, etc., may be used.

at least fifteen minutes in the steam. After removing the cleaning solution from the test tubes:

1. Wash them in water with a test-tube brush, using cleaning powder if necessary.

2. Rinse with tap water till clean and free from cleaning powder.

3. Rinse with distilled water.

4. Drain.

5. Test tubes and other glassware, flasks, pipettes, etc., may be rinsed with alcohol to facilitate drying, then drained.

Flasks. After treating flasks with cleaning solution:

1. Wash them as clean as possible with tap water and a flask brush; use cleaning powder if necessary. (When using cleaning powder, empty all water out of the flask, wet the flask brush with tap water, dip it in the cleaning powder and then rub the soiled portions vigorously.)

2. Rinse with tap water till clear and free from cleaning powder.

3. Rinse with distilled water and drain.

Petri Dishes. After removing Petri dishes from the cleaning solution:

1. Wash them in water, using cleaning powder if necessary.

2. Rinse with tap water. (It is not necessary to use alcohol or distilled water.)

3. Wipe immediately with a clean physician's cloth.

Pipettes. 1. Place pipettes delivery end down, in a glass cylinder (graduate) in cleaning solution and allow them to stand over night. (Steam may break the glass cylinder.)

2. Pipettes which have been used should be washed immediately. Grease which cannot be removed with water should be treated with 10% NaOH* and then with cleaning solution.

3. Rinse with tap water, followed by distilled water.

* If strong NaOH stands in contact with a poor grade of glassware for a length of time, it etches the glass, leaving a blue haze which cannot be removed.

4. Rinse with alcohol. (Alcohol may be used repeatedly.)

5. Drain.

Fermentation Tubes. 1. Rinse with tap water.

2. Fill with cleaning solution and heat fifteen minutes in steam or allow to stand over night if more convenient.

3. Wash thoroughly in tap water, using a test-tube brush if necessary.

4. Rinse in distilled water and drain.

Cover-glasses and Slides. 1. Immerse the cover-glasses or slides, *one by one* in a 10% solution of sodium hydrate (NaOH) for thirty minutes only. This strength of NaOH will etch the glassware if left longer.

2. Wash *separately* in tap water, *handling with ordinary forceps*.*

3. Put, *one at a time*, in cleaning solution, and leave over night as convenient.

4. Wash *separately* in water.

5. Immerse in *clean* alcohol (95%).

6. Wipe with a *clean* physician's cloth.

7. Store in clean Esmarch and deep culture dishes respectively, to keep free from dust.

Other Glassware. Some modification of these methods will be adaptable to nearly all glassware.

Note 1. Glassware containing liquefiable solid media is best cleaned by heating and pouring out the material while in liquid condition, then treating as above. (Solid media when liquefied by heat should never be thrown in the sink, as it will solidify when cold and clog up the traps and drains.)

Note 2. Flasks, test tubes, Petri dishes, etc., *containing cultures*, must be heated one hour in flowing steam *before cleaning*.

Cultures containing spores should be *autoclaved previous to cleaning*.

Note 3. If cultures or media have become dry, add water before heating.

Especially care must be used in cleaning glassware in which mercuric chloride or any other disinfectant has been used.

* Always handle cover-glasses and slides with forceps.

STERILIZATION

Sterilization consists in the destruction of all forms of life. It may be effected by various agents. As applied to the practical requirements of the bacteriological laboratory many of those agents such as electricity, sunlight, etc., are of little value and are limited in their applications; others are so well suited to particular purposes that their use is almost entirely restricted to such applications.

The Two General Methods of Sterilization are:

A. Physical.

1. Plasmolysis or Plasmoptysis.
2. Desiccation.
3. Heat—(a) dry heat; (b) moist heat.
4. Light.
5. Filtration.
6. Dialysis.
7. Comminution.

B. Chemical.

1. Disinfectants, etc.

A. PHYSICAL

I. Concentrated solutions destroy microorganisms by withdrawing water from their cells (*plasmolysis*), e.g., in the preservation of food by concentrated salt or sugar solutions.

Microorganisms accustomed to a concentrated nutrient substrate may suffer *plasmoptysis* (bursting of the cell) if placed in a less concentrated medium.

In either case, if they are subjected gradually to the changing conditions, death is delayed or prevented.

II. Desiccation is destructive to many microbes, especially to those which do not form spores. For example, *Ps. radiculicola* is very sensitive to desiccation on the ordinary cover-glass or on cotton.

III. Sterilization by Dry Heat.

1. Sterilization in a naked flame.
2. Sterilization in an ether flame.
3. Sterilization in a muffle furnace.
4. Sterilization by hot air.

1. *Sterilization in a Naked Flame.* (a) The simplest means of sterilizing a metal instrument is to heat it to redness in a flame. This method is always adopted for sterilizing platinum, copper, etc., wires and iron and nickel spatulas, forceps, etc.

A platinum needle should always be carefully dried before sterilization, by holding it near the flame. This avoids sputtering, which scatters microorganisms, especially if moist material, e.g., fat or protein, on the needle is immediately thrust into the flame.

(b) An instrument may be sterilized by flaming it, i.e., by passing it rapidly through a hot flame. This method is useful for instruments, etc., having polished surfaces devoid of creases in which microorganisms might escape destruction, e.g., knives, glass rods, handles of platinum needles, mouths of test tubes, flasks, pipettes, etc.

(c) Deep wounds are sterilized by cautery with an instrument heated to a dull red heat.

2. *Sterilization in an Ether Flame.* In an emergency, small instruments, needles, etc., may be sterilized by dipping them in ether or absolute alcohol and after removal lighting the adherent fluid and allowing it to burn off the surface of the instruments. Repeat the process. It may then be safely assumed that the apparatus so treated is sterile.

3. *Sterilization in a Muffle Furnace.* Porcelain filter candles are sterilized by heating them to white heat in the muffle furnace. This method of sterilization cannot be applied to porcelain filters with metal fittings, such as Berkefeld filters.

The destruction of autopsied animals and accumulated

wastes of the laboratory is also best accomplished in this manner.

4. *Sterilization by Hot Air.* Exposure to hot air is the usual method of sterilizing all glassware, instruments with metal handles, etc., but it is not suitable for organic substances, with the exception of wool, cotton and paper.

To insure efficient sterilization, the prepared glassware,

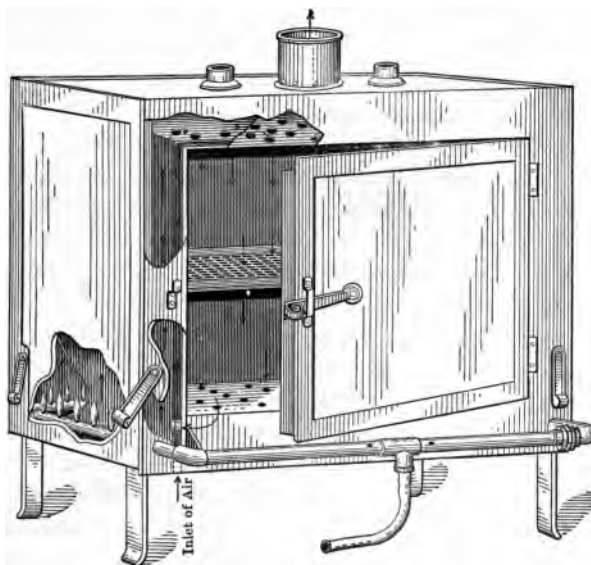


FIG. 2.—Hot Air Sterilizer.

etc., must be placed in a gas or electrically heated oven (containing a thermometer registering over 200° C.) whose temperature is maintained at approximately 150° C. for one hour, or 180° C. for ten minutes. The oven must be allowed to cool down to 60° C. before opening the door to avoid the breaking of glassware by cold-air currents. Cotton, wool, and paper are slightly scorched at this temperature.

Apparatus must be absolutely clean and *dry* before being sterilized.

IV. Sterilization by Moist Heat. Sterilization by moist heat may be effected in one of four ways:

1. By continuous or discontinuous heating at low temperatures (56°–80° C.).

2. By continuous or discontinuous heating in water at 100° C.

3. By continuous or discontinuous heating in flowing steam at 100° C.

4. By one heating in superheated steam (steam under pressure) at temperatures above 100° C., generally 115° C. (about 10 lbs. pressure) or 120° C. (about 15 lbs.).

1. *Sterilization by Continuous or Discontinuous Heating at Low Temperatures.* Some substances used as culture media, being rich in volatile or otherwise chemically unstable substances, cannot be heated to 100° C. without a marked alteration (e.g., coagulation) and to some extent a destruction of their properties; blood serum, for example.

Pasteur showed that such media can be better sterilized by heating them at a low temperature (55°–60° C.) for a long time than at a high temperature (70° C. or even 100° C.) for a short time. In this process, heat is not applied directly, as a rule. Control of the temperature is ordinarily accomplished by means of water heated to the degree desired.

Prolonged heating at a low temperature constitutes **pasteurization**. In practice, however, it is found that in order to kill all organisms pasteurization must be combined with the method of discontinuous heating devised by Tyndall. Albuminous media subjected to the Tyndall method must be incubated finally at 37° C. for forty-eight hours to eliminate all contaminated specimens.

2. *Sterilization by Continuous or Discontinuous Heating in Water at 100° C.* (a) *Continuous Heating.* Water at 100° C. destroys the vegetative forms of bacteria almost

instantaneously, and spores in from five to fifteen minutes ordinarily, although many spores of resistant species are not killed by several hours' heating at 100°C . Water suspected of sewage contamination may thus be rendered safe for drinking purposes simply by boiling for a few minutes.

This method is applicable to metal instruments, syringes, rubber stoppers, rubber and glass tubing, and other small apparatus.

(b) *Discontinuous Heating*. (Tyndall method.) Tyndall observed that certain resistant forms found in an infusion made from hay were not destroyed by heating the infusion at 100°C ., once, even when the temperature was sustained for a prolonged period, yet by boiling it for a short time on three successive days all living organisms were destroyed. His theory was that by heating at 100°C ., the vegetative forms but not the spores were killed. The latter germinate as the fluid cools and are killed during the second heating. A few spores, however, escape destruction at the second heating; these will have germinated by the time the third heating is due. After the third heating sterilization is accomplished.

The explanation now given, however, is that the resistance of spores is gradually lowered under the influence of repeated heatings. This principle of heating, on three successive days, a medium to be sterilized is now known as the **Tyndall method of sterilization**. In general laboratory practice, steam is used instead of water at 100°C ., but this necessitates special apparatus, whereas water lends itself readily to the needs at hand.

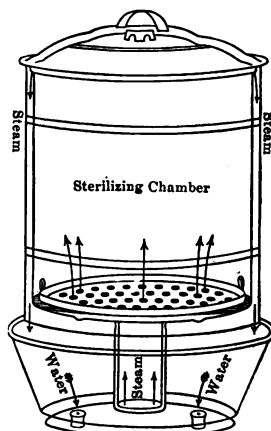


FIG. 3.—Arnold Steam Sterilizer.

The physical nature or bulk of the medium, the extraordinary resistance of the spores of certain bacteria or both in combination, may require that this intermittent heating be carried on over a longer period of time, i.e., four, five, six, etc., days in succession for the same or a longer period each time, or that the period between intermittent heatings be lengthened from twenty-four hours to forty-eight hours.

Tyndall's method is valuable in that media of delicate composition may be sterilized without producing undesirable changes, such as are often produced by the high temperature of the autoclav.

3. *Sterilization in Flowing Steam at 100° C. Continuous or Discontinuous.* (a) *Continuous Heating.* Simple boiling or exposure to steam at 100° C., even though the exposure be prolonged, is not a reliable method of sterilization. When microorganisms have been dried, their resistance to the effects of heat is much enhanced, and especially is this the case when they are mixed with substances of a colloidal nature. Certain resistant forms of protoplasm known as spores may not be destroyed by one heating to 100° C., even when the temperature has been maintained for several minutes.

(b) *Discontinuous Heating.* General use for the sterilization of media.

This principle of sterilization advanced by Tyndall finds its widest application in bacteriological work with the use of flowing steam. High-pressure steam may be utilized to good advantage if a central heating station is available. The Arnold sterilizer makes use of steam for the sterilization process and lends itself readily to both the continuous and discontinuous method (Fig. 3).

4. *Sterilization by Superheated Steam* (under pressure and therefore above 100° C.). Water, syringes, surgical dressings, bedding, india-rubber apparatus, filters, old cultivations, culture media, etc., not injured by high tem-

peratures, may be more quickly sterilized by heating in steam under pressure.

Exposure to steam at a temperature of 115°C . for twenty minutes is in most cases sufficient to insure sterilization,

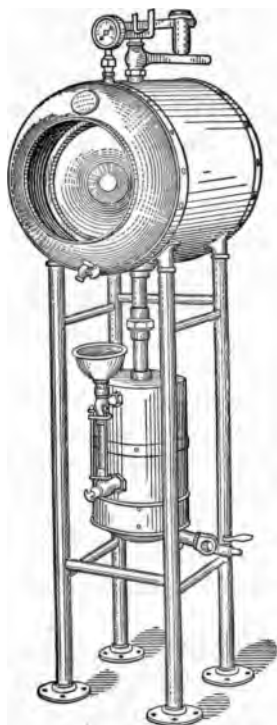


FIG. 4.—Autoclav, Horizontal,
for Steam or Gas.

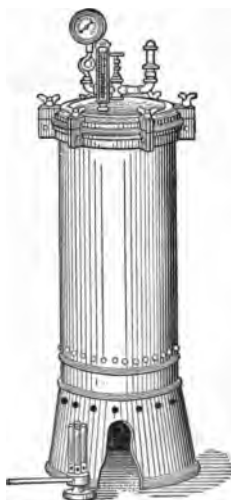


FIG. 5.—Autoclav, Vertical,
for Gas Only.

but some media, potato for instance, require a temperature of 120°C . for ten to fifteen minutes. It is now realized that media subjected to this high temperature undergo hydrolytic changes which render them unsuitable for the cultivation of more delicate microorganisms. Sterilization

in the superheated steam is carried on in a special apparatus called an **autoclav**, which may be so constructed as to run by direct or indirect steam. The latter is the more desirable for the sterilization of media.

V. Sterilization by Light. Light seems to act by producing powerful chemical germicides, probably organic peroxides, in the medium surrounding the bacteria. Certain rays of light, the blue, violet and ultraviolet in particular are destructive to living cells. It is to these rays that sunlight owes its disinfecting action. Practical use has been made of the ultraviolet rays in water sterilization by employing the Cooper-Hewitt mercury vapor lamp having a quartz instead of a glass tube, as these rays do not pass through glass.

VI. Sterilization by Filtration. Sterilization may be effected by the filtration of gases or liquids through materials which will retain microorganisms.

The best example of the filtration of gases is the use of cotton plugs in flasks and tubes containing microorganisms. The cotton is porous enough to allow the necessary interchange of gases but will allow neither dust nor foreign microorganisms to enter. The sterilization of air or other gases if forced through cotton would depend upon the thickness of the cotton layer and also upon the force which was exerted.

Certain fluids used in bacteriological work cannot be subjected even to a moderate amount of heat without profoundly altering their nature. In order to make such a fluid sterile, it is passed through a cylindrical vessel, closed at one end like a test tube, and made either of porous "biscuit" porcelain, hard burnt and unglazed (Chamberland filter) or of kieselguhr, a fine diatomaceous earth (Berkefeld filter) and termed a **bougie** or **candle**. (Fig. 70, p. 314.)

The pores of the finer filters are so small that, while liquids and solids in solution pass through, microorganisms are retained and the liquid passes through in a germ-free

condition. Pasteur in his early work utilized plaster plates as the filtering medium, but as a result of Chamberland's researches, porous porcelain now supersedes plaster. Finely shredded asbestos packed tightly in a Gooch crucible will serve as a bacterial filter provided the layer of asbestos is sufficiently thick. The rate of filtration is usually very slow because the pores of the filter are so very minute; therefore to overcome this disadvantage either aspiration or pressure is generally employed to hasten the process. This method may not exclude filterable organisms.

VII. Sterilization by Dialysis. In one of the more recent methods devised for the preparation of antirabic vaccines the vaccine is prepared by placing the virus (spinal cord of a rabid rabbit) in a collodion sac and dialyzing it in running distilled water. The living virus is destroyed, yet its immunizing properties are retained unimpaired. Quite the opposite effect may be obtained under somewhat different circumstances. If a collodion sac containing a suspension of a pathogenic organism be placed in the body cavity of a susceptible animal the organisms within the sac thrive, being nourished by the body fluids which diffuse through the semi-permeable membrane.

CUMMING, J. G.: Rabies—Hydrophobia. A study of fixed virus, determination of the M. L. D., vaccine treatment (Högyes, Pasteuf, and dialyzed vaccine), and immunity tests. *Journal of Infectious Diseases*, Vol. XIV (1914), pp. 33–52.

VIII. Commminution or the actual crushing of the microbial cells is resorted to for demonstrating intracellular enzymes.

B. CHEMICAL

I. Sterilization by Disinfectants. Sterilization by disinfectants has but limited use in bacteriological work. The amount of disinfectant necessary to destroy existing organisms in a nutrient medium is greater than the amount necessary to inhibit multiplication of an organism which may subsequently be used as an inoculum; the medium is therefore rendered useless.

1. Disinfectants may be used for any apparatus which will not come in direct contact with culture media or with the organisms under investigation. Fixed non-volatile disinfectants must be employed, since the vapors given off by volatile compounds hinder the growth of organisms on culture media.

2. Disinfectants are in general use for sterilizing the hands, woodwork, for washing out vessels and sterilizing instruments during inoculation and other experiments.

As an example, 1-1000 mercuric chloride, 1.5% formalin, 5% phenol, 2% compound solution of cresol, etc., are cheap and adaptable in many cases. Tincture of iodine is valuable for painting wounds.

The common *soaps*, and more particularly green soap, have a slight germicidal value, and this in conjunction with their solvent action upon fats and protein, and the mechanical cleansing which accompanies their use, justifies assigning them an important place among the chemical disinfectants.

Disinfectants used for sterilizing the skin before collecting pus, blood, etc., from the living subject must be carefully removed by washing the part well with alcohol before collecting material, otherwise the presence of the disinfectant would materially interfere with the subsequent growth of organisms in the culture.

3. Disinfectants are also added to sterile filtrates which are no longer required as culture media. For this pur-

pose a small quantity of some disinfectant (such as thymol or camphor) which is without chemical action on the constituents of the fluid is selected.

An amount of carbolic acid (0.5%) or other chemical is frequently added to vaccines, bacterins, serums, etc., for preservative purposes.

4. Disinfectants are sometimes used to sterilize a culture when the products of the microorganisms are under investigation. Chloroform, ether, toluol, oil of garlic or mustard, etc., which may be driven off afterward by evaporation, are among the most useful in this connection.

II. Sterilization by Antiseptics. Chemical reagents such as belong to the class known as antiseptics, i.e., substances which inhibit the growth of, but do not destroy bacterial life, are obviously useless.

REFERENCES

- EYRE: Bacteriological Technic. Second Edition (1913), pp. 26-48.
BESSON: Practical Bacteriology, Microbiology, and Serum Therapy (1913), pp. 3-27.
MARSHALL: Microbiology (1911), pp. 64-67.
EULER: General Chemistry of the Enzymes (1912), pp. 118-123.

EXERCISE 2. PREPARATION OF GLASSWARE FOR STERILIZATION

The mouths of test tubes, fermentation tubes, pipettes, etc., are ordinarily plugged with cotton before sterilization. For this purpose cotton is ideal as it is cheap and adaptable, serves to filter out microorganisms from the air, while allowing the ready diffusion of gases, and after once used it may be burned.

Paper (ordinary newspaper) may be used to wrap glassware as Petri dishes, deep-culture dishes, pipettes, etc., which one wishes to store in a sterile condition and for which cotton is not adaptable.

Glassware is sterilized for the purpose of destroying

microorganisms present on its surface and in or on the cotton or paper used respectively for plugging or wrapping. After sterilization the cotton and paper serve to prevent microorganisms from entering and contaminating the sterile utensils.

Dry heat, though not as effective a germ destroyer as moist heat, is more adaptable to the sterilization of empty culture flasks, pipettes and other glassware. Hot-air sterilization not only accomplishes the sterilization of the glassware, cotton plugs, etc., but "sets" the plugs so that they may be handled with greater facility.

All glassware must be absolutely clean and dry or contain *traces of alcohol only* before preparing for sterilization; otherwise sterilization cannot be accomplished. If considerable moisture is present in test tubes, flasks, etc., it will not evaporate during the hot-air sterilization process, and it is very evident that the temperature of such moist portions of the glassware will not reach or at least will not exceed 100° C.

Directions. Test tubes and flasks are plugged with cotton. The ordinary forceps are used for this purpose. (A glass rod may also be used.) A small piece of cotton is grasped on the edge with the forceps and inserted in the mouth of the test tube. Plugs should project into test tubes from 3 to 4 cms., and from 3 to 5 cms. into the neck of flasks, according to the size of the flask. Only an amount of cotton should project out of the mouth that is sufficient to protect the outward turned portion (lip) of the test tubes or flasks from dust. A "Christmas-tree" effect is to be avoided. Plugs should not be so tight as to be removed with difficulty, nor so loose as to offer no resistance to removal. A little experience will suffice to demonstrate the amount of cotton to use and the firmness with which the plug should fit.

Cotton plugs for test tubes, flasks, etc., may be *rolled*. This kind of plug is more stable and may be used several

times. Have the instructor demonstrate the method of rolling.

For hot-air sterilization, test tubes plugged with cotton may be tied in large bundles or placed in wire baskets

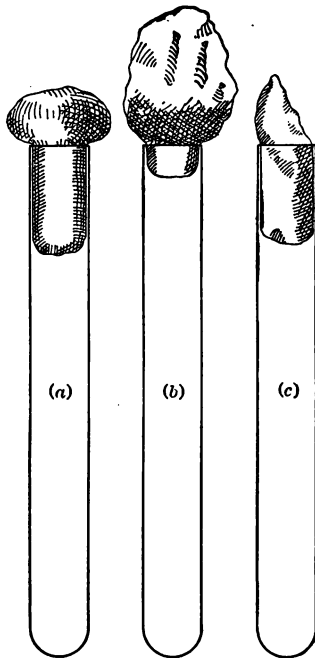


FIG. 6.—(a) Proper Plug; (b) Plug too Shallow and too Loose, too Much Projecting; (c) Plug too Loose, too Little Projecting.



FIG. 7.—Copper Cylinder for Sterilizing Pipettes.

(never in agate cups), *cotton plugs up*. A few test tubes should not be placed in a large wire-basket or in a wire test-tube rack, as it is necessary to economize space in the hot-air sterilizer.

Petri dishes are wrapped separately in paper and tied together in sets of three. One sheet of newspaper makes

four papers of proper size for wrapping Petri dishes and is inexpensive.

Three or more Petri dishes may be wrapped together *if all are to be used at the same time*. Mark each plainly with the desk number.

Pipettes. Place a piece of cotton in the bottom of a test tube, plug the top only of the pipette with cotton (*not too tightly*), leaving but little of the cotton projecting out. Wrap a small portion of cotton around the lower third of the pipette, insert the pipette into the test tube until the tip rests on the cotton, making the cotton wrapping serve as a plug for the tube.

Wrap pipettes so prepared in paper (one layer) and tie and mark them plainly with the desk number.

A covered metal case is often used for holding pipettes to be sterilized. The upper end of the pipettes are plugged with cotton, the pipette inserted in the case, the open end of the case plugged with cotton, and the cover replaced. (This latter method is not recommended for the new student, as the necessity of careful technic in removing a sterile pipette from the case without contaminating those remaining is difficult to impress upon him).

Fermentation tubes are plugged with cotton as directed for test tubes; the cotton plug should not project into the bulb.

Deep culture dishes are wrapped singly in paper as directed for Petri dishes.

Slides and cover-glasses are generally sterilized by flaming, but *only as needed*.

NUTRIENT MEDIA

“Chemically, like all other living cells, microorganisms consist of organic and inorganic nitrogen and mineral salts; it is therefore necessary in order to grow a microorganism, that these three classes of substances be made available, together with oxygen, which is an essential to the life of all

living structures. Finally a certain amount of moisture is absolutely necessary." (Besson.)

A food prepared for the growth of microorganisms is given the general term **nutrient medium**. A large number of microorganisms will grow readily in or upon easily available nutrient media, as milk, bouillon, etc. Some microorganisms have widely differing food requirements and need for growth nutrient media differing widely in their composition.

However, there are a few general rules that must be applied in the preparation of all nutrient media for the use of microorganisms. These are briefly, that: Every culture medium must—1. Contain substances necessary for growth. 2. Be of suitable reaction. 3. Be contained in vessels which afford protection from contamination from without.

Classification of Nutrient Media. Culture media may be classified as:

I. *Natural Media*—as occurring in nature, e.g., milk, potato and other vegetables, meat and meat products, blood and blood serum, egg, soil, etc.

II. *Prepared media*, i.e., made in the laboratory. These are:

(a) Of unknown chemical composition; e.g., nutrient agar, gelatin, etc.

(b) Synthetic; i.e., chemical composition known, e.g., Giltay solution for denitrifying organisms.

Or as:

I. *Liquid Media*. These include:

A. *Media made from animal tissue and fluids*, e.g., nutrient broth, serum broth, carbohydrate broths, milk, blood, nitrate peptone solution, Dunham's solution.

B. *Media made from vegetable tissue*. Among these are: Malt extract (germinated barley), beer wort, yeast extract, hay infusion, natural fruit juices, wines (fermented fruit juices).

C. *Synthetic media*.

II. *Solid Media*. These may be classified as:

A. *Liquefiable*, e.g., nutrient agar, nutrient gelatin.

B. *Non-liquefiable*, including: 1. Media liquid in a natural state but which, once solidified, cannot be liquefied by physical means, e.g., media prepared from albuminous fluids and tissues such as egg, blood serum, etc., or synthetic media solidified with sodium silicate.

2. Media which are solid in the natural state, e.g., vegetable media such as potato, carrot, banana, etc.

EXERCISE 3. TITRATION OF MEDIA

The titration of bacteriological media made from meat is an important step in their preparation, as microorganisms are sensitive to the reaction of the nutrient substrate.

Procedure. The following method is used for laboratory media, with the exception of milk, wort, cider, vinegar, fruit juices, etc. See p. 22.

1. Put 5 c.c. of the medium to be tested and 45 c.c. of distilled water in an evaporating dish.

2. Boil briskly one minute with constant stirring (to drive off all dissolved CO₂ which registers as acidity).

3. Add 1 c.c. phenolphthalein solution for indicator.

4. Titrate while hot, preferably while boiling, with N/20 sodium hydroxide, or N/20 hydrochloric acid as the case demands. A faint but distinct permanent rose color marks the end point. *This color should remain permanent for five minutes.*

5. Compute and record the reaction of the medium in degrees of Fuller's scale, which is the number of cubic centimeters of normal* acid or alkali present in 1000 cubic

* A solution is said to be normal when it contains 1 gram equivalent of an acid or base in 1 liter.

A gram equivalent of an acid or a base is that quantity which is equivalent to or will neutralize 1 gram molecule of a mono-basic acid or of a mon-acid base.

The advantage of the system is that 1 c.c. of any normal solution will exactly neutralize or be exactly equivalent to 1 milligram equivalent of any acid or base. (Noyes, Wm. A., Textbook of Chemistry, 1913, p. 184.)

centimeters of the medium, using phenolphthalein as indicator.

6. Alkaline media are denoted by placing a minus (-) sign before the number of degrees of alkalinity; thus, -15° would indicate that the medium was 15° alkaline, or that 15 c.c. normal acid must be added per liter to neutralize it.

Acid media are denoted by placing a plus (+) sign before the number of degrees of acidity; thus, $+15^{\circ}$ would indicate that the medium was 15° acid or that 15 c.c. of normal alkali must be added per liter to neutralize it.

Example.

Burette reading after titrating.....	5.4 c.c.
Burette reading before titrating.....	2.0 c.c.
Number of c.c. N/20 NaOH required ———	
to neutralize the acid in 5 c.c. of	
the medium	3.4 c.c.

If 5 c.c. of the medium (which is $1/20$ of 100 c.c.) require 3.4 c.c. of $1/20$ normal NaOH to neutralize the acid present, 100 c.c. of the medium would require 20×3.4 c.c. or 68 c.c. of $1/20$ normal NaOH.

As a normal solution is 20 times the strength of a $1/20$ normal solution, 100 c.c. of the medium would require $1/20$ of 68 c.c. or 3.4 c.c. of normal NaOH for neutralization; and one liter or 1000 c.c. of medium would require 10×3.4 c.c. or 34 c.c. N/1 NaOH for neutralization; i.e., *the medium is 34° acid, Fuller's scale.* This is the *titre* of the medium.

When N/20 *acid or alkali* and a 5 c.c. portion of medium (in 45 c.c. of distilled water) are used, each $1/10$ of 1 c.c. corresponds to 1° Fuller's scale.

Adjustment of Reaction. If it is desired to leave the medium with a, e.g., $+15^{\circ}$ reaction, we have:

Acidity of the medium

(+34°) 3.4 c.c. per 100 c.c. of the medium

Desired acidity (+15°) .. 1.5 c.c. per 100 c.c. of the medium

Amount of *normal* alkali ———

to be added. 1.9 c.c. per 100 c.c. of the medium

or $10 \times 1.9 \text{ c.c.} = 19 \text{ c.c. N/1 NaOH per 1000 c.c. of medium}$

Since normal solutions are of equal strength by volume, that is, 1 c.c. of N/1 acid will just neutralize 1 c.c. of N/1 alkali, it will readily be seen that if 15 c.c. N/1 NaOH are required to neutralize the acid present in 1 liter of medium, then there must be present in that liter exactly 15 c.c. of N/1 acid, or we should say the reaction is (+15°) fifteen degrees acid. For any other degree of acidity add enough normal alkali to reduce the acidity to the point desired.

The *reaction of a medium changes somewhat after its neutralization, especially during sterilization*, but also upon standing afterward at ordinary temperature. This change is toward an increased acidity, and is most marked in media rich in dextrose. Consequently it is necessary to determine the titre of a medium *at the time it is used* rather than to quote figures obtained before sterilization.

MILK, CIDER, VINEGAR, WORT, AND FRUIT JUICES

Procedure. 1. Into an evaporating dish measure 5 c.c. of the medium to be tested, by means of suitable pipette. Make up to 50 c.c. with distilled water.

Do not heat. The above media should not be heated before titration, as they contain volatile acids or other organic substances which may register as acid and which may be driven off by boiling.

2. Add 1 c.c. phenolphthalein solution.

3. Add, gradually, from an accurate burette, N/20 NaOH until the *first permanent pink appears*.

4. Note the amount of NaOH required for the titration.

5. Always run duplicates.

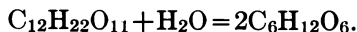
6. Record as degrees of acidity the number of c.c. of N/1 NaOH which would be required to neutralize one liter of medium.

MILK

Milk is valuable as a nutrient medium for microorganisms because: It is a natural nutriment and almost ideal for a large number of microorganisms. Its composition, averaging 3.40% fat, 3.50% casein and albumen, 4.50% milk sugar, 0.75% ash, 87.75% water, is an evidence that it furnishes food in an excellent form for most microorganisms.

The biochemical activities of many bacteria reveal themselves definitely in the changes which milk, especially litmus milk, undergoes. Many of these changes are seen macroscopically. Some of these are:

(a) **Acid Production.** The lactose, $C_{12}H_{22}O_{11}$ (milk sugar), is first inverted, forming two hexose molecules, 1 mol. dextrose and 1 mol. galactose.



And each molecule of hexose yields two molecules of lactic acid:

hexose = lactic acid.



The blue litmus is turned red.

(b) **Alkali Production.** Litmus becomes darker blue. This change very often accompanies peptonization.

(c) **Reduction (Decolorization of Litmus).** This is due to the reduction of the coloring matter (litmus). Many microorganisms secrete enzymes which produce hydrogen. The hydrogen combines with the litmus, reducing it to its leuco-compound (colorless). (Methylen blue becomes colorless under like conditions.) That this is a reduction and not a destruction may be demonstrated by shaking the decolorized culture with a few cubic centimeters of hydrogen peroxid. The bacteria which decolorize the litmus also

reduce the hydrogen peroxid to H_2O and nascent oxygen which reoxidizes the reduced litmus (showing by the reaction of the milk the type of microorganisms present). Re-oxidation takes place slowly under natural conditions. Reduction may take place when milk is acid, alkaline or neutral.

(d) **Curdling through Acid Production.** The casein, like most proteins, is **amphoteric**, i.e., it is capable of reacting both as a weak acid and a weak base. The otherwise insoluble casein is found to be in the milk in a partially dissolved state (colloidal), due to its combination with the calcium salts: the calcium that was formerly combined with the casein, through the production of acid by certain microorganisms, now combines with the lactic acid; as a result the casein precipitates, causing curdling (coagulation). Litmus is turned decidedly red. Milk having an acid curd will titrate above $+50^\circ$.

(e) **Rennet Curd.** Coagulation may also take place when the medium is neutral or only slightly acid. This production of curd is due to a rennet-like enzyme produced by microorganisms, and is similar to the action of the rennet used to curdle milk in cheese factories.

Many spore-forming species are found under the group of rennet-producing organisms. Rennet curd is usually followed by peptonization.

(f) **Peptonization.** The curd produced by acid or rennet-forming microorganisms may gradually disappear, leaving only a whey-like liquid. This is caused by certain bacteria which produce proteolytic enzymes that digest the curd and render it soluble. This liquefaction of solid proteins like gelatin, fibrin, boiled egg white, milk curd, etc., is due to two groups of enzymes, *pepsin* and *trypsin*.

The pepsin of the animal body acts only in an acid medium (present in the stomach).

The trypsin of the animal body acts only in alkaline medium (present in the intestine).

The pepsin- and trypsin-like enzymes produced by microorganisms cannot be thus separated by their activity in a medium of certain reaction; this varies with the species of microorganism and with environmental conditions. Peptonization of milk usually takes place in a neutral, slightly alkaline, or more infrequently slightly acid reaction.

Some organisms peptonize milk without forming a rennet curd.

(g) **Gas Production.** This is characterized by the formation of gas bubbles in the milk, and is generally accompanied by the formation of acid curd. Very commonly the curd shrinks, causing **extrusion of whey**.

EXERCISE 4. PREPARATION OF LITMUS MILK

Apparatus. Fresh separated or skimmed milk; titration apparatus; N/20 NaOH; phenolphthalein (indicator); 5 c.c. pipette; azolitmin, 2% solution; filling funnel; pinch cock; sterile test tubes; apparatus for steam sterilization.

Method. 1. Fresh separated or skimmed milk should be used. Whole milk is undesirable on account of its fat content.

2. Titrate and record the reaction of the milk. If the milk titrates above 17° acid, the reaction must be adjusted to +15°. Sour, curdled or uncurdled milk, after neutralization, does not make a desirable nutrient medium for microorganisms, therefore, milk whose titre is above 20°–25° acid should be discarded.

Fresh milk varies in acidity from 12° to 18°. Milk with an acidity above 18° to phenolphthalein will not give a satisfactory blue color with azolitmin, as at 18° it begins to show the acid coloration.

3. Add 2% of a standard solution of Kahlbaum's azolitmin. Litmus or azolitmin is added merely as an indicator and should be of sufficient strength so as not to dilute the milk to any extent.

4. Mix the milk and the azolitmin thoroughly and tube, using approximately 8 c.c. of the litmus milk in each tube.

Note. Care should be taken to prevent the milk from coming in contact with the top of the tubes, as it will cause the cotton fibers to adhere to the tube. This may be avoided by the use of a "filling funnel."

5. Sterilize by heating in flowing steam for twenty minutes on four successive days. Milk is difficult to sterilize, owing to the resistant spores which are frequently present. If it is desired to sterilize a larger bulk than in tubes, the time of heating should be lengthened.

Caution: Overheating tends to change (caramelize) the milk sugar. The color of the azolitmin may also be destroyed. These changes are not desirable.

EXERCISE 5. PREPARATION OF GLYCERIN POTATO

A number of chromogenic and pathogenic organisms thrive especially well on media containing glycerin. The manner in which glycerin favors the growth of these organisms is not known, but in some instances it seems to be directly utilized for the construction of fat (*Bact. tuberculosis*).

Apparatus. Large healthy potatoes; cylindrical potato knife, or cork borer; ordinary knife; tumbler; sodium carbonate, 1 : 1000 solution; glycerin, 5% solution; large sterile test tubes, or Roux potato tubes; absorbent cotton or short glass rod; 1 c.c. pipette; distilled water; apparatus for steam sterilization.

Method. 1. Carefully clean one or two large potatoes.

2. By means of a cylindrical potato knife or cork borer, cut cylinders of potato, 4 to 6 cm. long and 1.5 to 1.8 cm. in diameter. With an ordinary knife, halve each cylinder by a diagonal cut so that each piece resembles in shape an agar slant. Remove any portions of the skin on these pieces.

3. Place in a tumbler and soak in a dilute (1 : 1000) solution of sodium carbonate* for twenty-four hours *only*.

*Sodium carbonate is used to neutralize the natural acids of the potato.

4. Transfer the pieces to a 5% solution of glycerin in water for a further twenty-four hours *only*.

5. Place in sterile tubes prepared as follows: Select extra large test tubes 1.5 to 2 cm. in diameter and clean and dry them. Place a small piece of absorbent cotton or glass rod 0.5 cm. \times 2.5 cm. in the bottom of each. Plug with cotton and sterilize in the usual way. (Roux tubes need only to be cleaned and sterilized.)

Just before introducing the pieces of potato, add about 1 c.c. of distilled water to each tube, using a pipette. The potato should not touch the water.

6. Sterilize by heating at 100° C. on *four* successive days for twenty minutes each day.

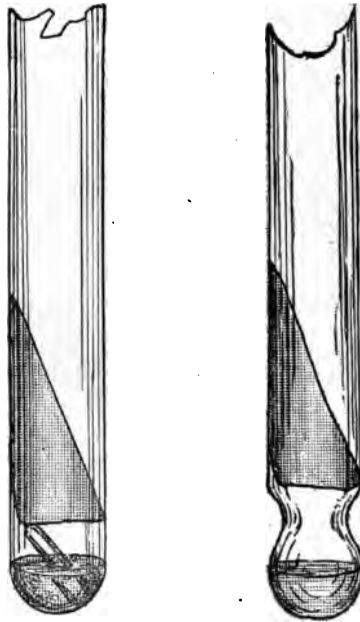


FIG. 8. — Potato Tubes. (Orig. Northrup.)

Caution: The time stated in 3 and 4 must be strictly adhered to, else the potatoes will have to be discarded on account of contamination with resistant spore-forming organisms.

REFERENCE

SMIRNOW, M. R.: The value of glycerinated potato as a culture medium. *Cent. f. Bakt., II Abt., Bd. 41*, p. 303.

EXERCISE 6. PREPARATION OF MEAT INFUSION

Meat infusion is the foundation of the ordinary nutrient media, as broth, gelatin and agar, and also of a large number of special nutrient media, as sugar broths, etc.

Under these directions sufficient meat infusion is prepared to make 1 liter each of nutrient broth, gelatin and agar.

Apparatus. 1.5 kilograms (3 lbs.) finely chopped fresh lean beef; 1500 c.c. tap water; 3.5 liter agateware pail; large funnel; ring stand; clean cloth; 1 liter measuring cup; three sterile 1 liter Erlenmeyer flasks; refrigerator; apparatus for steam sterilization (autoclav preferable).

Method. 1. To 1.5 kilograms of finely chopped, fresh lean beef in a 3.5 liter agateware pail, add 1500 c.c. of tap water,* mix thoroughly and allow to stand in a cool place (refrigerator preferred) for sixteen to twenty-four hours *only*.

2. Set up a large funnel in a ring stand and place a piece of clean cloth in the funnel. Place a measuring cup under the funnel.

3. Strain the meat infusion through clean cheesecloth, thoroughly pressing out all the juice. 1.5 liters should be recovered. If any loss occurs make up to 1500 c.c., using tap water.

This resulting sanguineous fluid contains the *soluble albumins* of the meat, the *soluble salts*, *extractives* and *coloring matter*, chiefly *hemoglobin*.

4. Place 500 c.c. of meat infusion in each of three sterile 1 liter Erlenmeyer flasks. Replace the plugs, and heat in the autoclav at 120° C. for thirty minutes. This is a safer procedure than heating for a longer time in flowing steam.

During this heating the albumins coagulable by heat are precipitated.

It has been found necessary and also more convenient to prepare and sterilize meat infusion before proceeding with the preparation of the different media in which it is used,

* Approximately 500 c.c. of water to each pound of meat.

on account of the resistant spore-forming organisms which are almost universally present in the chopped meat; economy of time also is a consideration. Unless sterilized immediately, meat infusion decomposes quickly owing to the abundance and diversity of the microflora acquired during the various processes of preparation for market.

Infusion made from freshly chopped lean beef will vary in acidity between $+15^{\circ}$ and $+25^{\circ}$ Fuller's scale. If the reaction is markedly lower or higher, microbial action is taking place, which is, or may be, injurious to the food value of the medium in which the meat infusion is used.

The infusion contains very little albuminous matter and consists chiefly of the *soluble salts of the muscle, certain extractives*, and altered coloring matters along with slight traces of protein not coagulated by heat.

EXERCISE 7. PREPARATION OF NUTRIENT BROTH

Nutrient broth is the standard liquid employed for cultivating microorganisms. It is practically a beef tea containing peptone. Peptone, a soluble protein not coagulable by heat, is added to replace the coagulated albuminous substances which precipitate when the meat infusion is sterilized. Salt is added to take the place of the phosphates and carbonates, some of which are precipitated on adjusting the acidity of the medium by sodium hydroxide.

The reaction of ordinary nutrient media is adjusted to about $+15^{\circ}$ with phenolphthalein as indicator, as it is found that most microorganisms grow best on a medium neutral or slightly alkaline to litmus.

When it is required that nutrient media be clear, egg albumen reduced to a smooth paste with water (or the well-beaten white of an egg) is added. By coagulation, the egg albumen removes mechanically all small particles in suspension which otherwise would pass through the filter paper.

This process is most efficient when the egg albumen coagulates slowly.

As egg albumen begins to coagulate at about 57° C. it is *absolutely imperative* for good results that the medium be cooled to 40° - 50° C. before the addition of egg albumen.

Although egg albumen contains small amounts of soluble matter not coagulable by heat, as sugar, extractives and mineral matter, all of which will serve as microbial food, its purpose in nutrient media is primarily for its clarifying action.

Apparatus. 500 c.c. sterile meat infusion; 500 c.c. tap water; 10 gms. peptone, Witte's; 5 gms. salt; 10 gms. egg albumen (or one egg); 3.5 liter agate-ware pail; titration apparatus; N 20 NaOH; N 1 NaOH; phenolphthalein (indicator); distilled water; 5 c.c. pipette; large stirring rod; coarse balances; large gas burner; large funnel; plaited filter paper; filling funnel; sterile test tubes; sterile 1 liter flask; apparatus for steam sterilization.

Method. 1. Put the contents of a flask of meat infusion (500 c.c.) in an agate pail and add 500 c.c. of tap water.

2. Add 1% of Witte's peptone and 0.5% of salt.

3. Add 10 gms. of egg albumen which has been well mixed with 100 c.c. of tap water. (Put the egg albumen in a tumbler and add enough water to form a paste. Stir until smooth. Then add the remaining water. One egg* well beaten may be substituted.) Mix all thoroughly.

4. Heat in flowing steam for forty-five minutes or in the autoclave at 120° C. for thirty minutes.

5. Titrate with N 20 NaOH.

6. Adjust the reaction of the medium to $+15^{\circ}$ with normal NaOH or normal HCl. Retitrate and adjust again if necessary.

7. Counterpoise and note the weight.

8. Boil fifteen minutes over a free flame, stirring constantly.

* It is not necessary to add water to the egg.

9. Counterpoise and restore any loss by evaporation with distilled water.

10. Filter while boiling hot through plaited filter paper just previously washed with 1/2 liter of boiling water.

11. Pass the filtrate through the same paper till it is bright and clear.

12. Fill thirty sterile test tubes, using approximately 8 c.c. of this medium for each tube. Put the remaining broth in a large, sterile flask.

13. Heat the test tubes and contents in flowing steam twenty minutes on three successive days.

14. To sterilize a large flask of broth, heat for twenty minutes four days in succession.

GELATIN

Gelatin is one of the tools of the microbiologist. As such, it serves two purposes: as a solid culture medium, a technical device by which the isolation of a single species of microorganism is made possible, and, to those organisms which secrete proteolytic enzymes, it serves as a nitrogenous food material.

Gelatin bears the distinction of being the first substance used for a solid culture medium. This medium was originated in 1882 by Robert Koch and has since revolutionized the science of microbiology. Prior to the introduction of solid media, the isolation of a single species of microorganism involved much difficulty and almost always a certain measure of uncertainty. To quote from Jordan: "It cannot be a mere coincidence that the great discoveries in bacteriology followed fast on the heels of this important technical improvement, and it is perhaps not too much to claim that the rise of bacteriology from a congeries of incomplete although important observations into the position of a modern biologic science should be dated from about this period (1882)."

Koch's first plates were made by pouring the liquefied

nutrient gelatin upon sterile, flat pieces of glass. The student on becoming familiar with the difficulties of preparing satisfactory plates with the use of the "Petri dish" will appreciate those met with in Koch's first gelatin plates.

Gelatin is a protein, i.e., a nitrogenous food material. It contains as its essential elements carbon, hydrogen, oxygen, and nitrogen (other elements, however, such as sulphur, phosphorus, etc., may be present). Its empirical formula according to Schützenberger and Bourgeois is $C_{46}H_{121}N_{23}O_{39}$, but such a formula only gives information of the chief constituents and allows one to form some idea of the huge size of the molecule; no idea of the structure of the molecule is given. However, by digesting with dilute sulphuric acid, gelatin breaks down in the same way as the proteins, yielding glycine, leucine and other fatty amino-acids.

Gelatin is an animal protein, but does not occur as gelatin in the animal tissues. It exists there as the albuminoid *collagen* which is the principal solid constituent of fibrous connective tissue, being found also, but in smaller percentage, in cartilage, bone and ligament. Collagen from these various sources is not identical in composition and gelatin varies correspondingly, e.g., gelatin from cartilage differs from that of other sources in that it contains a lower percentage of nitrogen.

Gelatin, the body resulting from the hydrolysis of collagen, is also an albuminoid. (Hofmeister regards this hydrolysis as proceeding according to the equation:



collagen + water = gelatin

but in dealing with substances of such variable composition, empirical formulæ of this kind have no great significance).

Commercially, it is prepared from certain kinds of bones and parts of skin. These are selected, washed and extracted

by water and with a dilute acid (hydrochloric), with relatively little exposure to heat, so that as few as possible of the fluid disintegration products of the stock are formed and the jellying power of the resultant solution is not destroyed.

The term gelatin is derived from the Latin verb *gelare*, to congeal, and calls to mind the principal attribute of this substance, that of its stiffening or jellying property.

Gelatin belongs to that interesting class of substances called colloids. It is a typical example of the class, and exhibits the characteristic properties of the class. Colloids, in marked contrast to crystalloids, do not crystallize, do not readily diffuse and are impermeable to each other. The ultimate particles of colloids are much smaller than what we would ordinarily term a physical subdivision, but rather larger than chemical molecules; the diameter of the smallest particles in a colloidal solution, e.g., red colloidal gold, which have been counted by means of the ultra-microscope, is 6 millimicrons or 6 thousandths of a micron. A micron is one thousandth of a millimeter. (Bacteria are much larger, the smallest visible by means of the ordinary microscope being from 0.3 to 1.0 micron in diameter.) Consequently their reactions stand midway between the physical and the chemical changes of matter, as may be seen by considering the properties of gelatin.

Gelatin will absorb a considerable quantity of warm water (it is almost insoluble in cold water) and swells up, yielding a jelly which, upon application of heat, melts to a viscous, sticky solution that gelatinizes again upon cooling. The name of *hydrogel* is applied to colloids showing this property. Ordinary gelatin media for microbiological work contain 12% to 15% gelatin. When dried at medium temperatures, gelatin can again be redissolved and redried indefinitely. From this property it is called a *reversible* colloid to distinguish it from other colloids which, when their physical state is once changed, are insoluble, e.g., casein and silicic acid.

If superdried at about $130^{\circ}\text{C}.$, or superheated when in the gelatinous state either for a short time at a temperature above $100^{\circ}\text{C}.$, or for a long time at $100^{\circ}\text{C}.$, as in intermittent sterilization, the gelatin is so modified that its redissolving or resolidifying power respectively is lost. In superdrying, the loss of the redissolving property is laid to the too close contact of the constituent particles, a change in the physical state; in the superheated gelatin, the loss of the resolidifying power is probably due to the disintegration of the gelatin molecule, a more purely chemical phenomenon. This loss of the gelatinizing property is also caused by the enzymic activities of many microorganisms and is also a disintegration process.

Gelatin possesses a liquefaction point which, however, varies considerably under different conditions. Ordinarily, media containing 12% to 15% gelatin will liquefy or melt at a temperature in the vicinity of 24° to $26^{\circ}\text{C}.$, solidifying again at 8° to $10^{\circ}\text{C}.$ to a clear, transparent jelly. As a consequence, gelatin media may be employed only for organisms which do not require a higher temperature than 22° to $24^{\circ}\text{C}.$ for development. Overheating in the process of preparation or sterilization will cause a considerable lowering of the liquefaction point, perhaps ultimately so low that the medium will be liquid at room temperature (20° to $24^{\circ}\text{C}.$). It will readily be seen how the latter gelatin medium could not handily be used for the isolation of organisms. A few data will assist in fixing this in mind.

The solidifying property of gelatin varies in inverse proportion with the time of heating during the process of sterilization; its liquefying point is lowered on an average of $2^{\circ}\text{C}.$ for each hour of heating at $100^{\circ}\text{C}.$ This makes clear why such care must be taken in the preparation of a gelatin medium, in the fractional sterilization of this medium in steam, and why immediate cooling is necessary after the process of its preparation.

Although temperatures above 100° C. are much more destructive to the solidifying property than that of 100° C., it is possible to sterilize a medium containing 12% to 15% of gelatin in the autoclav (7 to 8 lbs. pressure) at 112° to 113° C. for twenty minutes or at 15 lbs. pressure (120° C. for five minutes) without impairing its usefulness as a solid culture medium.

This use of steam under pressure (dry steam) is almost necessary in the case of a gelatin medium to effect sterilization, since gelatin, from its source, method of preparation, and later liabilities to contamination, is almost certain to contain or bear upon its surface a large number of very resistant spores. Heating at 100° C. for thirty minutes on three or even four or five consecutive days is not always efficient, as these spores do not always germinate within twenty-four hours after heating and, referring to the data above, it is readily seen that the lowering of the liquefaction point is not to be considered as negligible in the process of intermittent sterilization.

Gelatin possesses another property which renders it valuable for bacteriological work: i.e., in gelatin plate cultures no water of condensation ordinarily collects on the cover of the Petri dish (as with agar) later to drop on the surface of the gelatin and thus obliterate forms of colonies and cause isolated colonies to become contaminated with neighboring ones. The storing of this medium either in test tubes or in plates, sterile or inoculated, is thus rendered much more simple than with agar.

REFERENCE

VAN DER HEIDE, C. C.: Gelatinöse Lösungen und Verflüssigungspunkt der Nährgelatine, Arch. f. Hyg., Bd. 30, 1897, pp. 82-115.

EXERCISE 8. PREPARATION OF NUTRIENT GELATIN

Apparatus. 500 c.c. sterile meat infusion; 500 c.c. tap water; 150 gms. gelatin; 10 gms. peptone, Witte's; 5 gms. salt; 10 gms. egg albumen (or one egg); water bath; thermometer; 3.5 liter agate ware pail; long heavy stirring-rod; titration apparatus; N/20 NaOH; N/1 NaOH; phenolphthalein (indicator); distilled water; coarse balances; large gas burner; large funnel; plaited filter paper; filling funnel; sterile test tubes; sterile 500 c.c. Erlenmeyer flasks; apparatus for steam sterilization; running-water bath or refrigerator.

Method. 1. Put the contents of a flask of meat infusion (500 c.c.) in an agate pail and add 500 c.c. of tap water.

2. Add 15% gelatin, 1% Witte's peptone, and 0.5% salt to the mixture.

3. Heat this mixture in a water bath to dissolve the gelatin, peptone and salt, stirring occasionally.

4. Cool to 40°–50° C. *This is imperative.*

5. Then add 10 gms. of egg albumen which has been well mixed with 100 c.c. of tap water. (Put the egg albumen in a tumbler, add enough water to form a paste and stir until smooth; then add the remaining water. One egg well beaten may be substituted.) Mix all thoroughly.

6. Heat in flowing steam for forty-five minutes or in the autoclav at 105° C. for thirty minutes.

7. Titrate with N/20 NaOH.

8. Adjust the reaction of the medium to +15° with normal NaOH or normal HCl. Retitrate and adjust again if necessary.

9. Counterpoise and note the weight.

10. Boil fifteen minutes over the free flame, stirring constantly.

11. Counterpoise and restore any loss by evaporation with distilled water.

12. Filter while *boiling hot* through plaited filter paper

just previously washed with 1/2 liter *boiling* water. Pass the filtrate through the *same* paper until it is bright and clear.

13. Fill thirty sterile test tubes, using approximately 8 c.c. of medium for each tube. Divide the remainder into two equal portions and place in sterile 1/2 liter Erlenmeyer flasks.

14. Heat in flowing steam twenty minutes on three successive days.

15. *Cool the gelatin in a running-water bath, immediately after each heating.* Care must be taken to heat the gelatin as little as possible, since part of the solidifying power of gelatin is lost with each application of heat.

16. To sterilize a large flask of nutrient gelatin, heat for twenty minutes on four days in succession.

AGAR

Agar or agar-agar (from a Malay word meaning "vegetable"), the substance which is used in preparing one kind of solid culture medium for bacteriological work, is a product prepared from various seaweeds found near the Indian Ocean and in Chinese and Japanese waters. This type of seaweed has several common names, as Ceylon or Jaffna moss, Bengal isinglass, etc. Various species are used for food and the trade is considerable.

Payen, a French chemist (about 1859), obtained the agar jelly from the seaweed, *Gelidium corneum*, in the following manner: The seaweed was allowed to stand for some time in a cold dilute solution of hydrochloric acid; the acid was removed by rinsing several times with water, then the seaweed was placed in a cold dilute solution of ammonia; next the ammonia was removed by repeated rinsing with cold water. During this process, the seaweed lost 53% of its weight in mineral salts, coloring matter, and organic constituents. The remaining portion was boiled in water,

during which process the vegetable jelly was extracted. The solution so obtained was poured off, leaving the useless sediment behind. This jelly is the same in composition as that existing in the vegetable tissues; it has not been changed chemically, as is collagen in the preparation of gelatin. The commercial agar is most probably prepared by evaporating this solution to dryness by different means.

Agar usually comes into the hands of the bacteriologist as long, slender, grayish-white strips, or as blocks, or more especially in recent years, in the form of a gray-white powder of European manufacture.

Agar, in contrast with gelatin, is a carbohydrate, i.e., it consists of a combination of carbon, hydrogen and oxygen only. Traces of nitrogen are present as impurities. The above qualitative determinations of its elementary constituents were made by Payen, by Parumbaru and by Hueppe, who made their determinations on agar from different sources. As far as can be ascertained, its empirical formula has not yet been investigated to any extent.

Like gelatin, however, agar is a reversible colloid. It soaks up in cold water, dissolves in hot water after a long boiling to a tasteless and odorless clear solution, and solidifies upon cooling to a more or less opaque jelly. Its watery solution is neutral or nearly neutral to phenolphthalein; still, a drop or two of twentieth normal sodium hydrate is sufficient to make the pink color perceptible.

The colloidal properties of agar are not destroyed by a long-continued heating at a high temperature, nor by the action of ordinary microorganisms as are those of gelatin. The above properties, however, are influenced and may be wholly impaired by the reaction of the liquid in which the agar is dissolved.

The reaction of the liquid, i.e., whether it is acid or alkaline, influences the agar as to its solubility, solidity, color, transparency, filterability and amount of condensation water. If agar is dissolved in a liquid of an acidity

equivalent to 0.1% HCl, the agar dissolves very readily, filters quickly, the resultant filtrate being a light yellow, transparent, slippery, watery solution which does not solidify upon cooling. If a smaller percentage of hydrochloric acid is used, solidification occurs (below 40° C.) but the jelly will not "stand up" and is therefore useless for agar slant or plate cultures. A large amount of condensation water is present also.

If agar is dissolved in a weak alkaline or neutral broth, a thick, reddish-brown, viscous liquid is obtained which filters slowly and solidifies quickly at 40° C., to a very solid, opaque, dry jelly, having but little condensation water; it retains its shape well in slants and in plates. Thus the value of the agar as a solid culture medium is raised or lowered according to the degree of alkalinity or acidity.

It must be noted in addition, however, that when once the solidifying property of agar is destroyed by the presence of an excess of acid in its solution, this property can never be regained by neutralization with alkali; the acid permanently destroys the reversibility of the colloid.

The melting-point of agar (of 1.5% in neutral solution) is 97° C. and although its solidifying point is at 40° C., when once it has solidified it will stand up in the thermostat at a temperature of 50° C. For bacteriological purposes, only that form of agar can be used which remains fluid at from 38° to 40° C. Agar which remains fluid only at a temperature above this point would be too hot when in a fluid state for use; the vitality of organisms introduced would be impaired or destroyed by the high temperature.

Difficulties are encountered in the preparation of a solid culture medium from agar, due to its slow solubility, viscosity and consequent slow filterability. Its solution (digestion) is effected, as mentioned above, by a long heating in a water-bath, steam sterilizer, autoclav, or over a free flame. The length of time required for complete digestion depends upon three things: The reaction of the

liquid in which the agar is dissolved, the per cent content of agar, and the method of dissolving. The influence of the reaction of agar solutions has been treated above. For general culture use, however, ordinary agar is made $+15^{\circ}$ Fuller's scale (agar solidifies with difficulty above $+30^{\circ}$ Fuller's scale).

One per cent agar is much more easily soluble under equal conditions than a higher per cent. One and one-half per cent is the amount used in ordinary agar media, giving a somewhat stiffer and thus more desirable jelly.

Agar is digested most rapidly over a free flame. If not heated sufficiently, after the filtration and sterilization of the agar by the intermittent method, a flocculent precipitate frequently appears in the previously clear medium. This can be made to disappear in most cases by subjecting to the temperature of the autoclav (120° C.—15 lbs.).

Agar for culture media should be entirely clear when liquid, and homogeneously opaque-translucent when solid; it should have a translucence sufficient to allow deep colonies on plates or stab cultures to be observed readily; it should not contain flocculent material, sediment, or pieces of cotton or filter paper, as these hinder typical colony development of microorganisms and, to the inexperienced, may sometimes be mistaken for colonies.

In the first methods ever used for making agar culture media, instead of filtering the hot agar through filter paper, absorbent cotton, or asbestos, it was allowed to cool, during which process the sediment settled to the bottom; when solid the sediment was cut off. This method was not desirable, as the clearness of the resultant agar would depend upon the rate of cooling; the slower the cooling, the more completely would sedimentation take place.

Agar is not a food for microorganisms in general, i.e., it is not affected by the digestive enzymes of most bacteria, as is gelatin. However, a few bacteria are known which have the power of liquefying agar, among which are *B.*

gelaticus n. sp. (gran) and *Bact. nenckii*, both of which are found, as would be expected, in sea water. This comparative inertness of agar renders it valuable for the preparation of solid synthetic media, the value of which may be enhanced by subjecting the commercial agar to natural fermentation during which process any traces of available food substances are used up by the microorganisms present. (Beijerinck.)

Agar is of special use in bacteriological work in which the cultivation of microorganisms must be conducted at a temperature above the melting-point of gelatin. This feature has made possible the great strides that have been taken in medical bacteriology, as many pathogenic bacteria can be isolated and grown only with difficulty at temperatures below that of the body.

REFERENCES

- SMITH, ERWIN F.: Bacteria in Relation to Plant Diseases. Vol. I, pp. 31-36. Several illustrations.
SCHULTZ, N. K.: Zur Frage von der Bereitung einiger Nährsubstrate. Cent. f. Bakt. I. Orig., Bd. 10, 1891, p. 57.

EXERCISE 9. PREPARATION OF NUTRIENT AGAR

Apparatus. 3.5 liter agate-ware pail; 15 gms. agar; 10 gms. peptone; 5 gms. salt; 10 gms. egg albumen (or one egg); 500 c.c. sterile meat infusion; 500 c.c. tap water; titration apparatus; N/20 NaOH; N/1 NaOH; phenolphthalein (indicator); distilled water; large funnel; plaited filter paper; filling funnel; sterile test tubes; sterile liter flask; coarse balances; large gas burner; 1 liter measuring cup; apparatus for steam sterilization.

Method. 1. In a 3 liter agate ware pail place 15 gms. of agar in 500 c.c. of tap water.

2. Wash the agar well, separating the shreds and squeezing it through the hands.

3. Decant the dirty water, measuring the amount poured

off; replace with the same amount of clean tap water. Repeat.

4. Dissolve over a free flame and boil for five minutes, stirring constantly. The solution must be *entirely free* from lumps of agar.

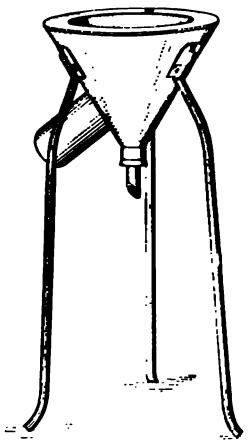


FIG. 9.—Hot Water Funnel for Filtering Agar or Gelatin.

5. Add 1% Witte's peptone and 0.5% salt to the boiling agar.

6. To 500 c.c. of meat infusion add 10 gms. of egg albumen which has been well mixed with 100 c.c. of tap water. (Put the egg albumen in a tumbler and add enough water to form a paste. Stir until smooth and then add the remaining water. One egg, well beaten, may be substituted.) Mix all thoroughly.

7. Pour the melted agar mixture slowly into the meat infusion, stirring constantly. Heat in the autoclav at 120° C. for forty-five minutes or for an hour in flowing steam.

Note. The time for this heating may be lengthened to advantage, but *never* shortened. If agar has not been heated sufficiently *before* filtration, a flocculent precipitate will form in the tubes upon heating in flowing steam. In most cases this may be caused to disappear by heating for a short time in the autoclav at 15 lbs.

8. Titrate with N/20 NaOH.
9. Adjust the reaction of the medium to +15° with normal NaOH or normal HCl. Retitrate and readjust the reaction if necessary.
10. Counterpoise and note the weight.
11. Boil fifteen minutes over a free flame, stirring constantly.

12. Counterpoise and make up any loss in weight with boiling distilled water.

13. Filter *boiling hot* through plaited filter paper *just previously washed with boiling water*. Pass the filtrate through the same paper until clear.

14. Fill 60 to 70 sterile test tubes, using approximately 8 c.c. of the medium for each tube.

15. Heat in flowing steam twenty minutes on three successive days.

16. At the end of the final heating, place the tubes of agar in an inclined position to solidify (do not allow the medium to touch the plug) so that a large surface is presented for the cultivation of microorganisms. These are called **agar slants**.

Note. If agar tubes are to be used only for agar slants, less of the medium is needed in the tube than when they are to be used for plating.

17. To sterilize a large flask of agar, heat for thirty minutes on four successive days.

EXERCISE 10. PREPARATION OF DUNHAM'S PEPTONE SOLUTION

Dunham's solution is utilized for determining the power of microorganisms to produce indol, ammonia or nitrites from peptone, which properties are characteristic of certain species.

Apparatus. 1000 c.c. of tap water; 10 gms. peptone, Witte's; 5 gms. salt; large burner; large funnel; plaited filter paper; filling funnel; sterile test tubes; apparatus for steam sterilization.

Method. 1. Mix 1% peptone and 0.5% salt to a smooth paste with a measured (small) amount of water.

2. Dilute to 1000 c.c. with tap water.

3. Counterpoise and note the weight.

4. Boil ten minutes over a free flame; counterpoise and make up any loss in weight with distilled water.

5. Filter while hot through a plaited filter previously washed with hot water. (Filtrate must be perfectly transparent.)

6. Tube, putting 8 c.c. in each tube.

7. Sterilize for fifteen minutes on three successive days.

Microorganisms which will not produce ammonia or nitrites from peptone may show this power if nitrogen is added to this solution in the form of inorganic nitrogen as potassium nitrate (0.2%).

EXERCISE 11. NITRATE PEPTONE SOLUTION

This solution is used to determine the power some organisms have of reducing nitrates to nitrites, free ammonia or nitrogen.

Apparatus. 1000 c.c. distilled water; 1 gm. peptone, Witte's; 0.2 gm. nitrite-free potassium nitrate; large agate-ware pail; filling funnel; sterile test tubes; apparatus for steam sterilization.

Method. 1. Mix the following ingredients: 1000 c.c. distilled water; 1 gm. Witte's, or other peptone; 0.2 gm. nitrite-free potassium nitrate. Filter if necessary.

2. Tube, placing 8 c.c. in each tube.

3. Sterilize by heating for fifteen minutes on three successive days or for five minutes in the autoclave at 120° C.

CULTURES

Definitions. A culture consists of the active growth of microorganisms in or on a nutrient medium.

A mixed culture is a culture composed of two or more species of microorganisms growing together in or on a nutrient medium.

A pure culture is the growth of one species of microorganism only, in or on a nutrient medium, that was sterile before inoculation.

Pure cultures are used for studying the morphological and physiological characteristics of microorganisms.

From **mixed cultures**, **pure** cultures may be obtained by the plating method. Mixed cultures of known microorganisms may be employed in studies on symbiosis, metabiosis, or antibiosis.

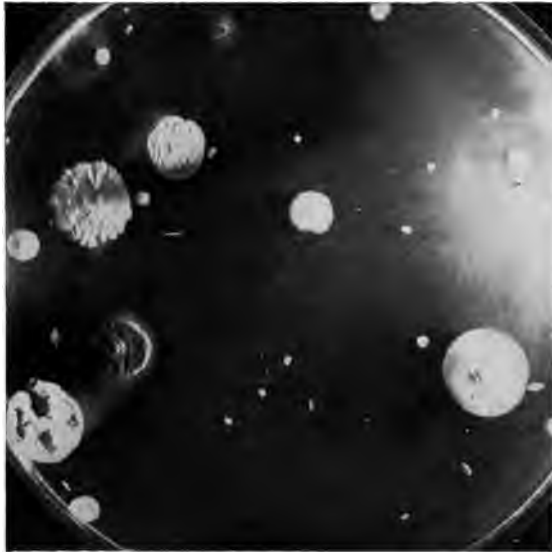


FIG. 10.—Mixed Culture in Petri Dish (Plate Culture) Showing Various Forms and Sizes of Colonies. (Orig. Northrup.)

Plate cultures are cultures grown in Petri dishes containing a nutrient medium.

Slant culture is the term generally applied to cultures grown on the inclined surface of any medium, such as agar, potato, blood serum, etc., and are designated specifically as *agar slant cultures*, *potato slant cultures*, etc. They are generally prepared by drawing a contaminated needle in a straight line along the surface of the medium. Cul-

tures prepared in this way are also frequently termed **streak cultures**. The term streak cultures may also be applied to cultures made similarly but grown on a horizontal flat surface as in a Petri dish.

Slant or streak cultures are valuable in offering a large surface for growth, to aerobic organisms.



FIG. 11.—Liquefaction of Gelatin, Saccate becoming Infundibuliform. (Orig. Northrup.)

Stab (or Stich) culture is the term applied to a culture, generally a pure culture, which is prepared by stabbing a translucent, liquefiable solid medium to a considerable depth with a contaminated straight needle. Gelatin stab cultures are invaluable for studying gelatin liquefaction. Agar is frequently used for stab cultures. If sugar is added to the medium, gas production may be demonstrated. Aerobic and anaerobic bacteria may be easily differentiated by their behavior in stab culture.

Liquid cultures are cultures grown in a liquid medium such as milk, broth, cider, wort, etc.

Shake cultures are made by inoculating with a pure or mixed culture, a liquefied nutrient medium (40° – 45° C.). The inoculum is distributed immediately throughout the medium by means of the needle used, or by rotating or shaking.

This type of cultivation is valuable for determining the oxygen relation of the organisms introduced and is especially useful for demonstrating the presence of gas-producing organisms if a suitable medium is used.

Care of Cultures. 1. *Incubation:* Cultures should be

kept at a constant temperature. Organisms which naturally grow at body temperature (37° C.) as *Bacillus coli*,



FIG. 12.—Petri Dish Rack and Battery Jar for Storing Media. (Orig.)

Streptococcus pyogenes, etc., may, with the exception of gelatin cultures, be kept in the 37° C. incubator.

Always place cultures in tumblers with cotton in the bottom or in small wire baskets; never place them in a

horizontal position or incline them carelessly against a vertical surface without proper support.

2. Care of Broken Cultures. If a culture of any organism is accidentally broken pour 1 : 1000 mercuric chloride, 2% compound solution of cresol or 5% phenol over it and also over any articles which may have been infected; *let stand ten minutes before wiping up.* Always disinfect your hands after handling broken cultures.

3. Disposal of Old Cultures. Heat glassware containing cultures to be discarded one hour in flowing steam. Cultures of pathogenic spore-forming organisms should be autoclaved. Glassware so treated may safely be washed by the student.

Never throw living cultures into waste crocks, sinks, or elsewhere. You safeguard yourself and others in the laboratory by destroying all living cultures. *Carelessness in regard to this matter will not be tolerated.*

4. Care of Slides, Cover-glasses, etc. Slides and cover-glasses used for hanging drop mounts, etc., should be immersed in 1 : 1000 mercuric chloride or chromic acid cleaning solution for at least ten minutes before cleaning.

5. Care of Cuts and Other Wounds. In case of cuts or wounds, consult the instructor at once. All wounds should be attended to immediately. Tincture of iodine is recommended for painting skin abrasions and deep wounds; in the latter case a bandage should be applied to keep extraneous matter from entering and setting up infection. In case of serious injury, a physician should be consulted. Every laboratory should keep a stock of rolled bandages, etc., for emergencies,

**EXERCISE 12. PREPARATION OF PLATE CULTURES,
LOOP OR STRAIGHT-NEEDLE DILUTION METHOD
(QUALITATIVE)**

Plate cultures are a valuable asset to the microbiologist, as they offer a means by which pure cultures of micro-organisms may most easily be obtained; they also allow a quantitative and qualitative study of the microflora of different substances.

Their preparation consists in, (1) inoculating a liquefied solid culture medium with micro-organisms, (2) mixing them well throughout the medium, (3) pouring the inoculated medium into a sterile Petri dish and, when it has solidified, (4) placing the Petri dish or plate culture at a constant temperature.

The culture medium in solidifying fixes *in situ* the micro-organisms introduced, and well-separated organisms develop into more or less well-separated "colonies" which become visible to the naked eye after twenty-four to forty-eight hours. From these isolated colonies usually pure cultures may then be obtained, or a quantitative or qualitative study may be made.

Isolated surface colonies are most frequently round (concentric in growth) and generally are quite typical for each species, while isolated sub-surface colonies are lenticu-



FIG. 13.—Water-bath for Melting Agar or Gelatin for Plating, containing a Removable Copper Test Tube Rack. (Orig. Northrup.)

lar (double concave) or compoundly lenticular in shape as a rule, species differences not being as well defined.

Apparatus. Tripod leveling stand; glass plate about



Fig. 14.—Pouring Plate. (Orig. Northrup.)

14 inches square; small spirit level; water-bath; thermometer; sterile Petri dishes; tubes of sterile media (gelatin or agar); culture; platinum needle and loop; Bunsen burner; wax pencil; mixed or pure culture.

I. Procedure for Agar Plates. The loop or straight-needle dilution method is valuable as a quick method of obtaining pure cultures when quantitative results are not desired.

1. Place the glass plate on the leveling stand.

2. Place the spirit level on the glass plate and make level by means of the leveling screws.

Note. The plate-leveling stand facilitates the uniform distribution of the medium over the bottom of the Petri dish, but is not necessary for the accomplishment of favorable results. If the desk top is level this apparatus is unnecessary.

3. Place three sterile Petri dishes, labeled 1, 2 and 3, in a row on the glass plate.

4. Liquefy three tubes of agar at 100° C. in the water-bath or steam and keep at a temperature of 40° to 45° C.

5. Number the tubes of agar 1, 2 and 3 and flame the plugs.

6. With the sterilized platinum needle, merely *touch* the culture and transfer to tube No. 1.

Note. Hold cultures and plugs while transferring as in Fig. 20, p. 59.

7. Distribute the microorganisms through the medium with the needle.

8. Transfer one loopful from tube No. 1 to tube No. 2 and mix with the needle, as in 7.

9. Slightly raise the cover of Petri dish No. 1. Introduce the flamed mouth of tube No. 1 and pour the melted agar into the plate; remove the mouth of the tube, and replace the cover of the Petri dish. If the medium has not entirely covered the bottom of the plate, tilt slightly in different directions to distribute evenly.

Note. Passing the Petri dish several times through the flame just previous to pouring the plate will aid greatly in preventing the formation of condensation water on the cover.

10. Transfer two loopfuls from tube No. 2 to tube No. 3 and mix.

11. Plate tube No. 2 in Petri dish No. 2 (see 9).

12. Plate tube No. 3.

13. Label the plates with *name of culture, number of dilution and date*, and with your own name or desk number.

14. When the agar has solidified firmly, invert the plates and place in the incubator at 37° C., or at room temperature.

Note. If the plates are placed right side up, condensation water forms on the cover and drops down upon the surface of the agar, causing the colonies to run together and thus destroying their characteristic appearance.

II. Procedure for Gelatin Plates.

1 3. Proceed as in I. "Procedure for Agar Plates."

4. Liquefy three tubes of gelatin in the water-bath and keep at a constant temperature of 30° to 35° C.

5 13. Proceed as in I. "Procedure for Agar Plates."

14. Place at a constant temperature of 21° C. The gelatin may not harden until placed at this temperature.

Note. Gelatin plates are kept *right* side up, as the organisms may liquefy the gelatin. The liquefied part would then fall from the medium upon the cover and ruin the plate for study.

EXERCISE 13. PREPARATION OF PLATE CULTURES, QUANTITATIVE DILUTION METHOD

In the method given below, "dilution flasks" are prepared containing measured amounts of water or salt solution in which a measured amount of the substance under investigation is placed.

As to whether water or salt solution is used depends upon the nature of the material to be dissolved or placed in suspension. If the substance whose microflora is to be studied contains a certain amount of various salts or other electrolytes in solution, an effort should be made to approx-

imate this amount in the preparation of the diluting fluid, e.g., in obtaining a quantitative estimation of the micro-

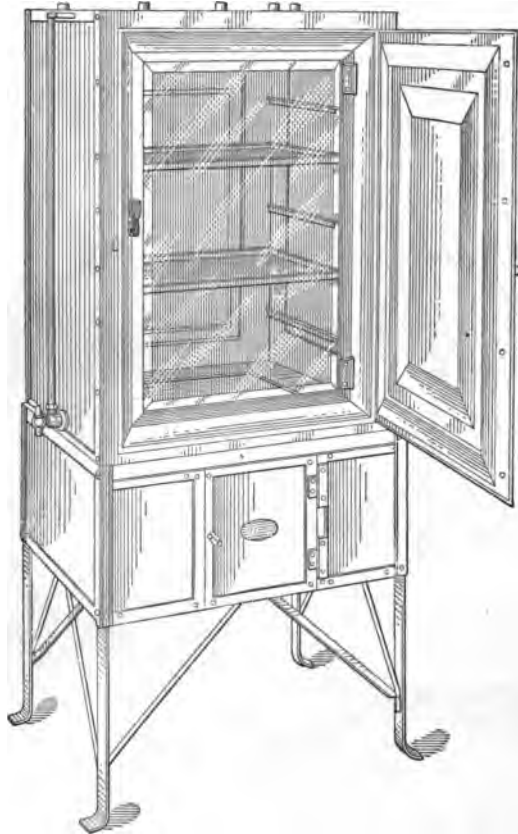


FIG. 15.—Incubator.

organisms from the blood, dilutions should be made in 0.85% salt solution; from tap water, in tap water, etc.

Theoretically, dilutions made in a liquid of a markedly different electrolyte concentration from that of the sub

stance to be studied, might cause either plasmolysis or plasmoptysis as the concentration was respectively too great or too weak.

Microorganisms of different species differ markedly in their susceptibility to osmotic pressure. This cannot be determined, however, unless studies are made of pure cultures of each, therefore the percentage of salt in the diluting liquid should approximate that of the substance whose microflora is to be studied.

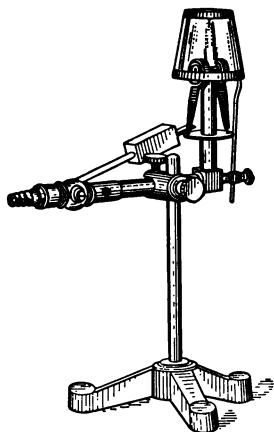


FIG. 16. — Koch's Safety Burner for Incubator or Water Bath.

The method below is applicable to substances in the liquid condition only. Modifications of this method may be utilized to apply to nearly every class of substances.

Plates are generally made from *three* different dilutions, so that well-separated colonies may be obtained on at least two plates.

Apparatus. Sterile 1 c.c. pipettes (graduated to 0.1 c.c.); sterile 10 c.c. pipettes (graduated); sterile Erlenmeyer flasks of 200 c.c. capacity containing 90 c.c. and 99 c.c. of sterile water or salt solution; three sterile Petri dishes; three tubes of sterile agar or gelatin.

Note. Use only freshly prepared dilution flasks, otherwise *evaporation* takes place so rapidly that accuracy is not possible.

Culture. Substance under investigation.

Method. 1. With a sterile 1 c.c. pipette, transfer 1 c.c. of the original sample or culture to a flask containing 99 c.c. of sterile water or salt solution. The flask now contains 100 c.c. of liquid containing 1 c.c. of the **original** sample, giving a dilution of 1 in 100.

Note. A sterile pipette must be used for each separate operation.

2. Shake the flask to secure an even suspension of the microorganisms.

Do not allow the liquid to touch the cotton plug.

3. With a sterile pipette, transfer 1 c.c. of the first dilution into a flask containing 99 c.c. of sterile water and shake. The second flask now contains 100 c.c. of a liquid containing 1/100 of the original sample, a dilution of 1/100 in 100, or 1 in 10,000.

4. If a higher dilution is required, 1 c.c. from the flask containing the 1/10,000 dilution placed in a flask containing 99 c.c. sterile water gives 100 c.c. of a liquid containing 1/10,000 of the original sample, or a dilution of 1 in 1,000,000.

If a lower dilution of the original sample than 1/100 is desired, make use of the 90 c.c. dilution flasks as follows:

With a sterile 10 c.c. pipette place 10 c.c. of the original sample into 90 c.c. of sterile water and shake. This flask now contains 100 c.c. of liquid containing 10 c.c. of the original sample, giving a dilution of 1 in 10. A dilution of 1 in 1000 may be made either by placing 1 c.c. of the 1/10 dilution in 99 c.c. of sterile water, or by placing 10 c.c. of the 1/100 dilution in 90 c.c. of sterile water.

Note. Almost any desired dilution can be made by the use of these flasks.

5. *For plating*, transfer 1 c.c. with a sterile 1 c.c. pipette from the flask containing the desired dilution to a sterile Petri dish.

Note. *Never use less than 1 c.c.* Run duplicates when absolute accuracy is necessary.

6. Liquefy the desired number of agar or gelatin tubes in the water-bath or steam at 100° C.

7. Cool to a temperature of 40° to 45° C.

8. Pour the plates, tilting each carefully so that the 1 c.c. of the diluted sample may be mixed well throughout the medium.

9. Place the plates on a level surface until the medium solidifies.

10. Incubate at the desired temperature.

EXERCISE 14. METHODS OF COUNTING COLONIES IN PETRI DISH CULTURES

Apparatus. Jeffer's counting plate; black glass plate or cardboard; tripod counting lens, magnifying four diameters; plate cultures.

Note. In Jeffer's counting plate (see illustration) each division has an area of 1 square centimeter. The figures denote the number of square centimeters in the respective circles.

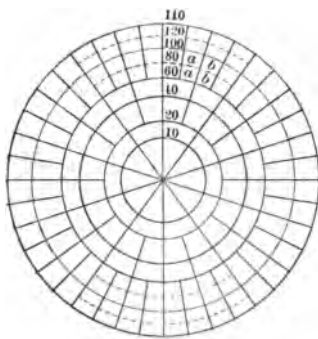


FIG. 17.—Jeffer's Counting Plate.

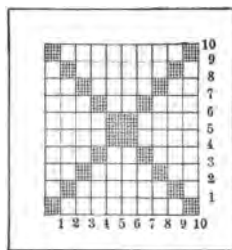


FIG. 18.—Wolfhügel's Counting Plate.

Method. 1. Invert the Petri dish culture to be counted upon the black glass plate or upon some black surface.

Note. If liquefiers are present on the gelatin plate, place the Petri dish right side up upon the counting plate; this necessitates refocusing the lens. The cover may be removed to facilitate counting if the plate is to be discarded.

2. Place the counting plate upon the Petri dish, making the circumference of the Petri dish coincide as nearly as

possible with that of one of the circles on the counting plate.

3. Using the tripod lens count the colonies in each sector of the smallest circle, then in each division between the concentric circles.

Note 1. The tripod counting lens must be used if the colonies are very small, as they otherwise may be confused with air bubbles in the medium. If there are less than 500 colonies present, the entire plate should be counted. If the number is much greater, from ten to twenty divisions, in some definite order, should be counted, an average taken, and the results multiplied by the area of the plate in square centimeters.

Note 2. Wolfhügel's counting plate is very desirable for counting a large number of colonies. It is ruled in square centimeters and the squares on the diagonals of the plates are subdivided into smaller squares. The colonies appearing in from ten to twenty of these smaller squares may be counted, an average taken and the result multiplied by the number of small squares in 1 sq. cm. times the area of the Petri dish in square centimeters. (The entire area of the plate may be obtained most quickly by placing a Jeffer plate upon the Petri dish in question.)

4. Ascertain the number of colonies per cubic centimeter in the original sample by multiplying the whole number of colonies on the plate by the dilution; e.g., if there are 386 colonies on the plate and the original culture was diluted 1 in 1000, the number of colonies contained in each cubic centimeter of the original sample is 386,000.

Note. When there is an excessive number of colonies on a plate the vigorous microorganisms will inhibit the growth of the less vigorous and thus the number of colonies counted is smaller than the number of microorganisms present. Moreover, the colonies may become confluent and the counts will again be in error.

EXERCISE 15. ISOLATION OF MICROORGANISMS FROM PLATE CULTURES AND METHOD OF MAKING AGAR STREAK CULTURE

Apparatus. Straight platinum needle; several tubes of sterile agar slants; Bunsen burner; wax pencil; plate containing from 30 to 200 well-separated colonies.

Method. 1. Examine the plate to determine the colonies which differ macroscopically and microscopically. (Use

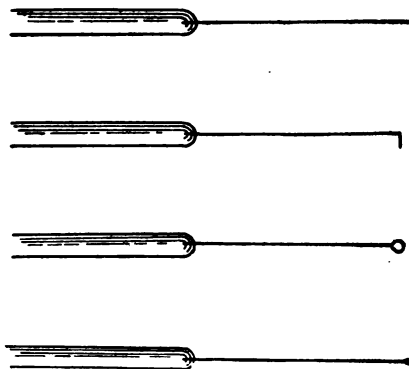


FIG. 19.—Various Forms of Platinum Needles. (Orig. Northrup.)

a counting lens or the lowest power of a compound microscope.)

2. Note the most isolated of each kind, and mark them with the wax pencil upon the bottom of the plate to insure picking up the proper colonies later. Also note how the deep and surface colonies differ.

3. Examine each marked colony under the lowest power of the microscope to make sure of its purity. If the colony does not appear to be wholly isolated, pick up a small portion of it with a sterile platinum needle and stain with one of the common stains (see p. 88) or examine it in the

hanging drop (see p. 76) to determine if more than one kind of organism is present.

4. If the colony is pure, pick up a portion with the



FIG. 20.—Transferring Cultures. (Orig. Northrup.)

sterile needle, or, in case of extremely small colonies, remove the cover from the plate, focus the low power of the microscope on the desired colony and while looking through the microscope, fish the colony.

5. Transfer to one of the agar slants, making a streak along the median line of the inclined surface of the agar, drawing the needle from the base to the top of the slant.

Note. For investigational purposes, when dealing with unknown microorganisms, the following method is more accurate for obtaining them in pure culture: Transfer to a tube of broth; incubate for twenty-four hours and plate a second time. Isolate from the twenty-four-hour plate culture.

6. Incubate at the optimum temperature.

Note. If the agar slants have become dried out to any extent, it is necessary that the agar be melted and re-slanted in order that optimum growth may take place.

EXERCISE 16. METHOD OF MAKING TRANSFERS OF PURE CULTURES INTO A LIQUID MEDIUM

Directions for making transfers of pure cultures from one medium to another must be followed *very carefully*, otherwise extraneous microorganisms may enter and hopeless confusion result.

Apparatus. Test tubes containing a sterile liquid nutrient medium; platinum needle; Bunsen burner.

Culture. Pure culture.

Method. 1. Flame the cotton plugs of the test tubes containing the pure culture and the sterile liquid nutrient medium.

2. Sterilize the platinum needle in the flame.
3. Permit it to cool (about one minute is required).
4. Hold it in the right hand and remove the cotton plug of the culture tube with the little finger of the same hand.
5. Take up a very little of the culture with the needle.
6. Replace the plug of the culture tube.
7. Remove the plug of the tube of sterile liquid medium in the same manner.
8. Insert the infected needle into the liquid.
9. Replace the plug.
10. Sterilize the needle before laying it down.

EXERCISE 17. METHOD OF MAKING STAB CULTURES

Apparatus. Tubes of sterile agar or gelatin; straight platinum needle; Bunsen burner.

Culture. Pure culture.

Method. 1. Liquefy the gelatin or agar tube and re-solidify it in a vertical position in cold running water or in some cold place.

2. With a sterilized *straight* platinum needle pick up a very little of the culture or colony.

3. Insert the needle at the middle of the circle made by the surface of the medium and push the needle about 5 cms. into the solid medium (within 1 cm. of the bottom of the tube), then withdraw carefully so that the path of the needle be as limited as possible. The microorganisms grow along the path of the needle.

Avoid having the shoulder of the rod come in contact with the surface of the medium lest its heat disfigure the surface or even kill the microorganisms. The surface of the medium should remain intact during this process.

4. Replace the plug in the new culture and sterilize the needle.

EXERCISE 18. PREPARATION OF A GIANT COLONY

Purpose. To show the development of a single colony of a microorganism.

Apparatus. Sterile Roux culture flask* or Petri dish; tubes of agar or gelatin.

Culture. Organism to be studied.

Method. 1. Melt two tubes of dextrose agar or gelatin. Pour into the culture flask.

Note. Allow the medium to touch and cover one large side only.

2. Heat in this horizontal position in flowing steam fifteen minutes.

3. Distribute the medium evenly over the large side,

* Most valuable for molds, especially *Rhizopus nigricans*.

and set on a level surface to cool. When the medium has solidified, place the flask with the medium-side up.

4. Mark the center of the flask on the outside with a



FIG. 21.—Giant Colony of Mold in Roux Flask. (Orig.)

wax pencil and inoculate with a bent platinum needle in one spot *only*.

Note. When making mold inoculations *moisten the sterile needle with sterile water or medium before touching the spores*; this insures a positive inoculation.

5. Keep at room temperature medium-side up.
6. Examine and measure the diameter of the giant colony from day to day and describe the typical growth

of the colony, using the terms on the descriptive chart of the Society of American Bacteriologists, p. 134, as far as possible.

7. Compare the giant colonies of a *Mucor*, *Pencilium*, a yeast and *Bacillus subtilis* or *Bacillus mycoides*. Use agar for giant colonies of these bacteria, as they liquefy gelatin.

8. Giant colonies of yeasts and bacteria and some molds may be grown in Petri dishes, or in flat-bottomed flasks.

For illustrations of giant colonies of bacteria see:

FUHRMAN: Vorlesungen über Technische Mykologie, pp. 41, 43.

LÖHNIS: Vorlesungen über Landwirtschaftliche Bakteriologie, pp. 38, 170.

LEHMANN AND NEUMANN: Bakteriologie und Bakteriologische Diagnostik, Bd. I. (Atlas.)

For illustrations of giant colonies of yeasts see:

LAFAR: Technische Mykologie, Bd. 4, German Ed., pp. 24-25, 306, and above references.

THE MICROSCOPE

Care of the Microscope. For microbiological work a compound microscope is necessary. This should be fitted with a minimum of two oculars corresponding to the Leitz No. 1 (lowest power) (Spencer, 6×), and No. 3 or 4 (Spencer, 10×) and three objectives corresponding to the Leitz $\frac{1}{4}$ in. (lowest power) (Spencer, 16 mm.), $\frac{1}{7}$ in. (Spencer, 4 mm.) objectives (dry) and $\frac{1}{2}$ in. oil immersion objective. A coarse and a fine adjustment permit the accurate focusing of any combination of lenses. The substage should be fitted with a good condenser and iris diaphragm for regulating the amount of light, and a plane-concave mirror.

Great care should be exercised in the use and care of the microscope as it is a delicately adjusted instrument.

The following rules should be heeded:

The Stand. The stand is the body of the microscope carrying the optical parts.

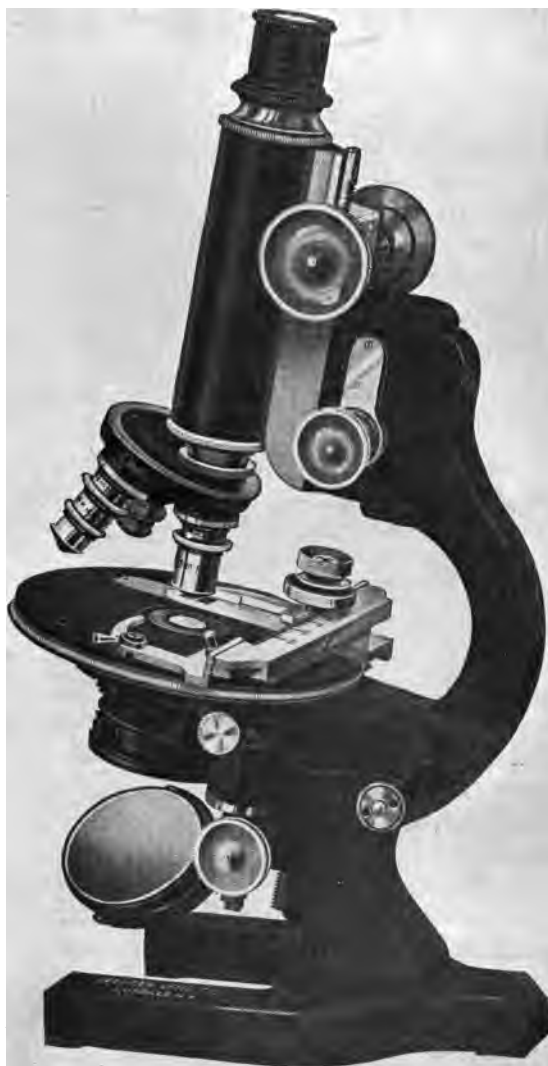


FIG. 22.—Compound Microscope with Mechanical Stage Attached and Side Fine Adjustment.

1. *Leave the microscope in the case when not in use.* Dust works into the bearings of the instrument, making them work hard and unnecessarily wearing them.

2. When handling the microscope do not grasp it by the arm which contains the fine adjustment unless the microscope is designed to permit this. Grasp it by the pillar below the stage.

3. *Never use alcohol on the lacquered parts.* Rubbing gently with a very little xylol and drying quickly will remove any oily material.

The Stage. The stage is that portion of the microscope on which the mounted object is placed for examination.

1. Should the stage become soiled with balsam, immersion oil or anything which water will not remove, it can be cleaned with xylol or chloroform. A little heavy oil will restore the stage to its original black color.

The Fine Adjustment. The fine adjustment is used for bringing out details in very small objects and is necessarily of limited range and delicate in its mechanism.

1. If, when looking into the eye-piece, no change of focus is noticed by turning the micrometer head, or if the micrometer head ceases to turn, the adjustment has reached its limit. To adjust, focus down or up, respectively, with the coarse adjustment, and turn the micrometer head until the fine adjustment is midway within its range.

2. When the fine adjustment screw stops, *do not force it.*

The Draw Tube. The draw tube is the tube receiving the ocular.

1. The draw tube should work easily and smoothly. On the draw tube will be found graduations in millimeters or inches, some fixed point at which certain combinations of objectives and oculars give the clearest image. This differs with different microscopes and should be known for the microscope used.

The Nose-piece. The triple nose-piece on the compound microscope serves a double purpose; to obviate the neces-

sity of screwing the different objectives in as needed and to protect the back lens of the objective from dust. The later microscopes have a "collar" nose-piece which keeps the objectives free from dust at all times.

1. Nose-pieces and objectives of the best makes are now made so that the objectives are *parfocal*, i.e., when one lens is in focus the others on the nose-piece will be nearly in focus when they are swung into the optical axis. They are also approximately centered so that a point in the center of the field of one lens will be in the field of the others.

2. Objectives made parfocal for one tube-length or eye-piece are not parfocal for a different length or a different eye-piece.

3. Objectives of one microscope should not be interchanged with those of another, even if of the same make.

4. *Always focus up*, slightly, before turning from a lower to a higher power. Otherwise the front of the objective may be swung against the cover-glass and injure both the specimen and the objective.

The Optical Parts. The optical parts are the lenses of the objectives, oculars and condenser and the mirror.

1. Wipe dirty lenses gently with Japanese lens paper to remove dirt.

2. Never rub a lens vigorously with anything.

3. Avoid touching the surface of a lens with the fingers. Cutaneous secretions are hard to remove.

4. Always clean the oil immersion objective with lens paper *immediately after using*. If the oil is allowed to dry, xylol must be used to clean the lens.

5. *Always leave an ocular in the tube* to keep dust from settling on the back lens of the objective. Dust on the back lens may be removed with a camel's hair brush.

6. *Never take an objective apart.*

7. Oculars, condenser and mirror should be kept clean by the use of lens paper.

Use of the Microscope. Position. 1. Always use the microscope with the tube in the perpendicular position. This is indispensable in examining fresh mounts or fluids.

2. *Work with both eyes open* and if possible use both eyes interchangeably.

Light. 3. *Never use direct sunlight.* The best light is obtained from white clouds. Northern or eastern light is preferable.

The best artificial light is a Welsbach burner (gas). When employing artificial light use a blue glass between

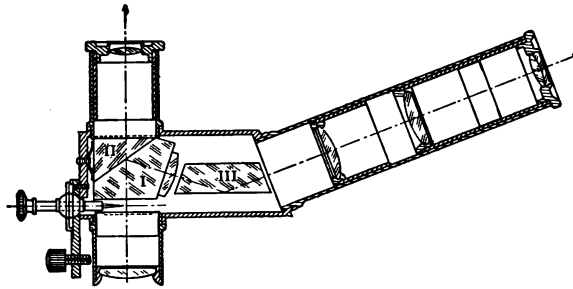


FIG. 23.—Double Demonstration Ocular with Pointer Enabling Two Observers to View Simultaneously the Image Indicated by the Adjustable Pointer.

the light source and the specimen. Often an eye-shade or some appliance with a similar purpose is desirable.

4. Use the plane mirror in daylight, the concave mirror with artificial light.

Focusing. 5. After putting in place a low-power ocular and objective, place the specimen on the stage, and while looking through the microscope, adjust the mirror so as to illuminate the field as evenly as possible, but not so brightly as to irritate the eyes.

6. By means of the coarse adjustment, focus the body tube until the objective *nearly* touches the cover-glass, *being careful not to touch it.*

7. With the eye at the ocular, focus up slowly with the coarse adjustment until the specimen comes plainly into view.

If the light is too intense the focal point may be passed without noticing it.

8. When the object is brought fairly well into focus by

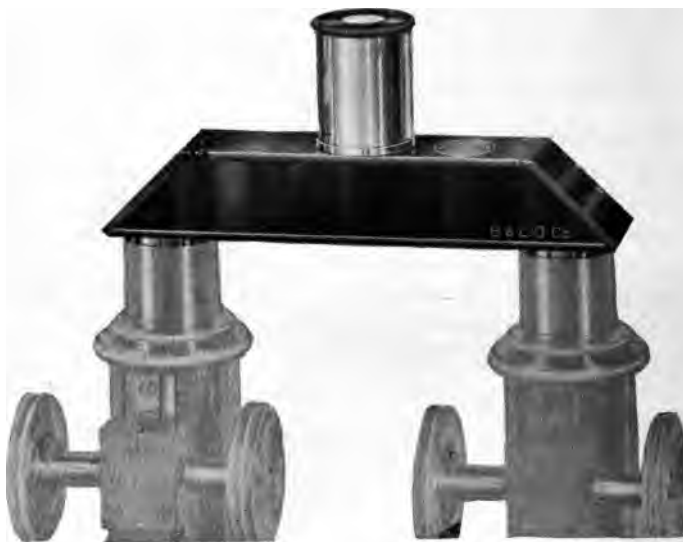


FIG. 24.—Comparison Ocular Enabling One to Observe Two Different Microscopic Fields Side by Side. Any two microscopes may be used, (Healy.)

means of the coarse adjustment, use the fine adjustment to obtain the sharpest focus to bring out details.

9. Move the specimen when trying to obtain a focus, as a moving object is more apt to be noticed as the lens comes into focus.

The microscope *reverses* the image. This will be noticed when the specimen is moved. The microscope *magnifies* the movement as well as the image; it therefore requires

a certain delicacy of movement to put a specimen in a desired position.

10. *Beginners should always use the low-power objectives and oculars first.* The low-power objectives have longer working distances and always show a larger portion of the specimen. After obtaining a general idea of the specimen, desired portions may be examined with the higher power objectives.

11. In using high-power objectives for finding and examining a specimen, it is always more desirable to use the lowest power ocular (corresponding to Leitz No. 1). If a higher ocular is used, there is a loss in the depth or sharpness and size of field, since they are both inversely proportional to the magnification. Illumination is also lost, which varies inversely as the square of the magnification. Remember that *the largest field, the greatest penetration, and the best illumination are obtained by using the lowest magnification which makes all the detail in the image visible.*

Oil Immersion Objective. The highest power objective is the oil immersion lens. This is so termed because a drop of oil must be used between the front lens and the cover-glass. The oil used must have the same index of refraction as glass to prevent the dispersion of the rays of light coming from the condenser.

Working distance is the free distance between the cover-glass and the objective when the latter is focused. High-power objectives have short working distances.

REFERENCE

GAGE: The Microscope.

EXERCISE 19. METHOD OF MEASURING MICRO-ORGANISMS

I. Using the Leitz Ocular "Step" Micrometer. In this ocular micrometer the intervals are arranged in groups of ten, each group being indicated by black steps rising from the first to the tenth interval.

This arrangement possesses the great advantage that the divisions can always be seen distinctly whether the objects be light or comparatively dark.

The intervals of the scale, instead of being 0.1 mm. or 0.5 mm. wide, as in ordinary ocular micrometers, have a definite value of 0.06 mm. This gives for each objective and for a given tube length, convenient and in many cases integral micrometer values, which renders a greater facility in the use of this instrument. The actual tube length differs in most cases but little from the standard length. The tube length and the micrometer value of each microscope, however, should be separately calibrated.

It is of importance to be able to determine the size of microorganisms: (1) because it is of general interest to know the size of the microorganisms with which we are dealing; (2) because the difference in size is an important factor in identifying and describing the organism; (3) because the size is necessary for purposes of comparison with other microorganisms.

Apparatus. Microscope; Leitz ocular "step" micrometer; object micrometer; specimen to be measured.

Method. 1. With the aid of the Leitz ocular "step" micrometer the size of stained or unstained microorganisms on either a light or a dark field may be measured directly in microns.

A **micron** is 0.001 mm., and is expressed by the Greek letter μ .

2. One hundred divisions of the step micrometer cover 100, 15 and 10 divisions of the object micrometer

when Leitz objectives 3, 7 and 1/12 oil immersion are used.

The object micrometer is simply a cover-glass (mounted on a slide in Canada balsam) upon whose surface has been ruled a scale 2 mm. in length, each millimeter being divided into 100 equal parts, the space between each division therefore being equal to 0.01 mm.

3. If 100 division lines of the ocular step micrometer cover 0.01 mm. of the object micrometer, then each division line of the step micrometer has the value 0.0001 mm. or 0.1 micron.

These values are only accurate when the draw-tube of the microscope is drawn out according to the following table.

4. *Using the ocular step micrometer and the object micrometer, find the tube length at which each objective gives a definite value in microns. This will vary some even with the Leitz oculars and objectives, so the tube length for each combination of lenses must be determined separately for any make of microscope.*

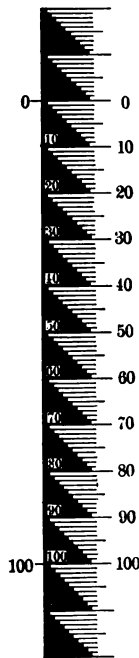


FIG. 25.—Micrometer Scale in Ocular of the Leitz Ocular Step Micrometer.

VALUES FOR LEITZ ACHROMATIC OBJECTIVES

No. of Objective.	Mark on Draw-tube.	Micrometer Value in Microns of Each Division Line of Step Micrometer.
3	141	10
7	174	1.5
1/12 oil imm.	150	1.0

5. *Multiply the number of division lines of the ocular micrometer covered by the organism in question by the value*

of the division line as determined in the above table. This gives the measurement directly in microns.

Microorganisms may be measured more accurately by mounting them in Chinese ink, as they cannot move, are not shrunk or distorted as often occurs with stained specimens, and are clearly seen. Preparations stained with

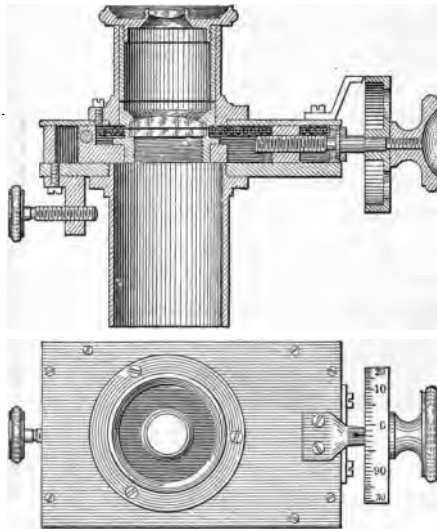


FIG. 26.—Ocular Filar Micrometer for Very Exact Measurements.

By means of a micrometer screw a line is moved across the field. The distance is measured by means of the divisions on the drum.

aqueous-alcoholic dyes stand next in preference, *never strong stains* like carbol-fuchsin, anilin-water dyes, or saturated alcoholic solutions of dyes.

II. Using a Filar Ocular Micrometer. The filar ocular micrometer is an instrument for the accurate measurement of microscopic objects. It consists of an ocular, between the eye and field lenses of which there is a scale ruled on glass in millimeters and half millimeters, below

and across which a single-line index is made to travel by the use of the micrometer screw.

The micrometer screw is fitted with a drum divided into 100 parts, one revolution of which moves the index line one division or 5 microns. The drum is divided into fifty parts, so that each mark on the drum scale corresponds to $\frac{5 \text{ microns}}{50}$ or 0.1 micron.

The micrometer value of each interval should be calibrated for each objective with the aid of the object micrometer. The eye-lens of the micrometer is adjustable to enable the observer to focus the scale accurately.

The filar ocular micrometer slips into the draw-tube of the microscope like any ordinary ocular and may be fixed in position by the milled-head screw on the side.

A. Calibration of the Filar Ocular Micrometer.

Apparatus. Filar ocular micrometer; microscope; object micrometer.

Method. 1. Place the object micrometer under objective No. 3 and ocular No. 1, drawing out the draw-tube to 17 mm.

2. Bring the lines on the object micrometer into a sharp focus.

3. Replace ocular No. 1 with the filar ocular micrometer.

4. Focus again so that the division of the object micrometer and the ocular micrometer are equally clear and turn the ocular micrometer so that the lines of both micrometers are parallel to each other.

5. Determine how many microns one space of the ocular micrometer represents.

Example. Six divisions of the ocular micrometer-scale cover the same length as three divisions of the object micrometer on which each division is 1/100 millimeter or 10 microns; therefore, six divisions of the ocular micrometer scale equals 30 microns and one division equals 1/6 of 30 or 5 microns.

6. Determine how many revolutions of the drum (from 0 to 0) are necessary to move the movable line one division and from this determination calculate the value in microns, of one division on the drum. One determination of this value is sufficient.

B. Method of Using the Filar Ocular Micrometer.

1. Replace the object micrometer by a slide containing organisms, focus, and measure an organism, counting the number of divisions the drum is turned in moving the movable line from end to end of the organism.

Example. If the drum is turned two divisions the organism was two times 0.1 micron in length or 0.2 micron.

2. To measure the microorganisms with a higher power objective, the value of each division of the scale has to be recalibrated.

EXERCISE 20. DETERMINATION OF THE RATE OF MOVEMENT OF MOTILE ORGANISMS

Apparatus. Microscope; Leitz "step" micrometer; stop-watch; hanging-drop preparation of motile organisms.

Method. Using a hanging-drop preparation of the organism to be examined, determine the rate of movement per second, using the step micrometer and a stop-watch.

EXERCISE 21. PREPARATION OF A HANGING DROP

The purpose of the hanging-drop preparation is to study bacteria in the living condition; to demonstrate (a) their form, (b) arrangement, (c) motility (this is best observed from twenty-four-hour cultures), (d) appearance, (e) division of cells, (f) formation or presence of spores; (g) to determine the presence and types of microorganisms in any material and to watch the changes in the predominating types of the microbial flora in a medium from day to day; (h) and, in pathogenic bacteriology, to demonstrate agglutination.

Bacteria have two kinds of movement, the so-called Brownian or *molecular movement*, and *true motility*. The former may be demonstrated by examining the movement of powdered carmen rubrum in the hanging drop. A very little of the powder is sufficient. Brownian movement is shown more or less by all small particles of insoluble matter (including living non-motile or dead bacteria) in suspension. It is characterized by a vibratory movement affecting the entire field; the relative positions of the insoluble particles are never altered. This type of move-

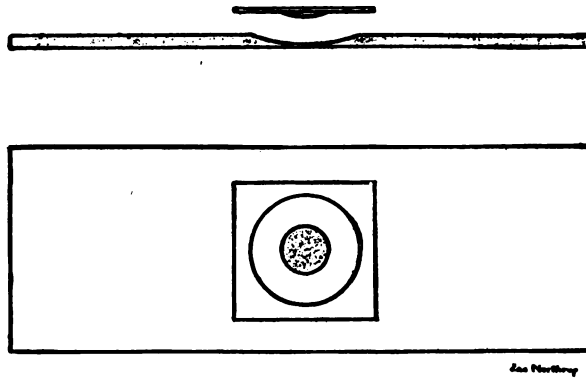


FIG. 27.—Hanging Drop Slide. (Orig.)

ment must be distinguished from that of true motility, which is characterized by the *progressive movement*, more or less rapid, of an organism across the field of the microscope, changing its position in the field independently of and in a direction contrary to other organisms present.

There should be no currents of air entering under the cover-glass and passing through the concavity of the slide nor should there be currents in the liquid. The latter may occur if the organisms have not been well mixed through the drop in the process of preparation. If large numbers of the microorganisms in the drop are moving *in one direc-*

tion, this is an indication of currents in the liquid which have been induced by the liquid touching the side of the concavity, by the drop being too large, by improper mixing, or by air currents; this fault may be remedied by thoroughly mixing the bacteria in the drop with the straight needle or by resealing the cover-glass upon the slide.

Apparatus. Clean cover-glasses; clean concave slides; platinum loop; straight platinum needle; Bunsen burner; distilled water; cover-glass forceps; melted paraffin or vaselin if preparation is to be sealed permanently.

Method. 1. With a platinum loop place four small drops of water about the edge of the depression of the concave slide.

2. For cultures:

In liquid media.

- (a) With a sterile platinum loop transfer a portion of the culture to the center of a clean cover-glass.

On solid media.

- (a) With a sterile platinum loop place a small drop of water or physiological salt solution in the center of a clean cover-glass.
- (b) With a sterile platinum needle transfer a minute portion of the culture to the drop of water so that only the *faintest* cloudiness appears.

3. Quickly invert the cover-glass over the depression in the concave slide and gently depress the margin on the water until the chamber is sealed air tight. The hanging drop must not touch the bottom of the concavity. Note the illustration. If it is desired to keep the hanging drop longer than five to ten minutes, it may be sealed with paraffin as with the adhesion culture, or with vaselin.

The drop must remain over the *center* of the concavity. If the drop touches the side of the concavity, the hanging drop as such is destroyed and it will be necessary to remake the preparation. If pathogenic organisms are used, both

slide and cover-glass must be placed in 1/1000 mercuric chloride or some equally efficient disinfectant for at least one hour before cleaning or reusing.

4. Examine first with objective No. 3, then with objective No. 7 or the 1/12 oil immersion lens, using ocular No. 1 in each case. After a perfect focus is obtained, ocular No. 4 may be used if desired.

Manipulation of Microscope. Using the lowest power objective and ocular, focus the tube of the microscope down by means of the coarse adjustment until the objective nearly touches the cover-glass, *being careful not to touch it*. Then, with the eye at the ocular, focus *up* with the coarse adjustment and move the preparation until the *edge* of the drop comes plainly into view. *This focal point may be passed without noticing it if the light is too intense or too dim*. The edge of the drop is a curved line. The preparation should be so moved that this line cuts the center of the field.

Focus up slightly, swing the No. 7 or 1/12 objective as desired, into place and after the field desired is obtained with the coarse adjustment, focus down until the objective nearly touches the cover-glass. Then with the eye at the ocular, focus up carefully with the coarse adjustment until the edge of the drop comes plainly into view. Use the fine adjustment to bring out details.

In using the 1/12 oil immersion lens a small drop of immersion oil is placed in the center of the cover-glass, the 1/12 objective swung into place as above. *Greater care must be exercised in focusing, as this objective has a shorter working distance.*

EXERCISE 22. PREPARATION OF THE ADHESION CULTURE

The purpose of the exercise is to show the germination of mold spores or the budding of yeast cells, i.e., colony formation.

Apparatus. Clean cover-glasses; clean concave slides; melted paraffin; small glass rod or camel's-hair brush;

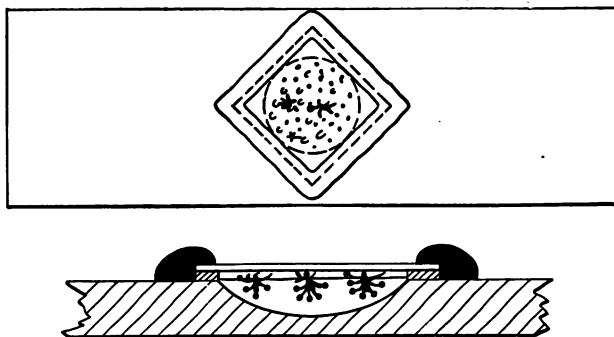


FIG. 28.—Lindner's Adhesion Culture. (Adapted from Lafar's *Technische Mykologie*.)

sterile cider in tubes. (Wort, milk and other media may be used as conditions demand.)

Cultures. Pure culture of mold, or yeast. If mold spores are to be germinated, an old culture having spores is necessary.

Method. 1. Inoculate a tube of sterile cider from the pure culture of the organisms to be studied. (Use spores in the case of mold and some of the cells for yeasts.) Distribute well with the platinum needle.

2. Transfer one loopful to a flamed cover-glass and spread in a thin film over the entire surface of the cover-glass, using the straight needle. If any of the cider adheres in droplets, shake them off.

3. Breathe into the concavity of a concave slide until small droplets of moisture are visible on the glass. Before this moisture evaporates and while the cover-glass is still wet turn the cover-glass, culture side down, cornerwise, covering the concavity on the slide.

4. Using the small glass rod or a camel's-hair brush dipped in hot paraffin, neatly seal the cover-glass on the slide so that the cavity will be air tight and the moisture will be retained. Success depends largely on quick work.

5. Examine with objective No. 7 and ocular No. 1. There should be five to twenty spores or cells on a slide. If more are found, a new culture should be made. It may be necessary to inoculate a second tube of cider from the first to secure the proper dilution.

6. If you are not familiar with the spores or cells of the organism to be studied, before making an adhesion culture, mount them in a drop of water heavily inoculated, cover with a cover-glass and examine microscopically.

7. Keep the cultures at room temperature. Examine as often as possible for thirty-six hours and then every twenty-four hours till growth ceases.

8. *Draw as many stages as possible.* The time required for spore germination is usually six to forty-eight hours.

Note. Some molds grow quite extensively in the adhesion culture, even producing fruiting bodies. *Very often both the mycelium and fruiting bodies show peculiar abnormalities and should never be drawn to represent normal structures.* These abnormalities are the result of the peculiar environment.

9. Failure to obtain growth of the mold spores or yeast cells may be due to imperfect sealing, insufficient moisture at the start or *too many cells* on the cover-glass. If the adhesion culture fails to grow, a fresh tube of cider must be inoculated before making new adhesion cultures, as the food materials contained in the medium are partly or entirely used. In the case of mold spores it is reasonable to expect that any mold spores in the adhesion culture

will have germinated within forty-eight hours after preparing the mount.

Note. This method may be utilized to study the colony development of bacteria also.

EXERCISE 23. PREPARATION OF THE MOIST-CHAMBER CULTURE

The purpose of the exercise is to study colony formation in molds, yeasts and bacteria.

Apparatus. Clean cover-glasses; small glass rings,

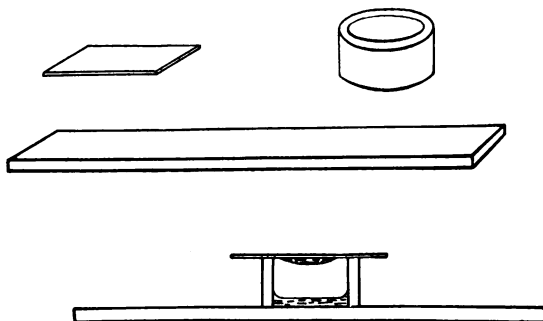


FIG. 29.—Moist Chamber Culture. (Orig.)

clean; clean slides; sterile pipette; paraffin or vaselin; sterile distilled water in test tube; tube of a sterile liquid nutrient medium; platinum needle and loop; forceps; cover-glass.

Culture. Pure culture of organism to be studied.

Method. 1. With forceps, carefully sterilize in a flame a glass slide and a glass ring designed for this purpose. This should be done by a swinging motion to insure uniform distribution of the heat.

2. Around the edges of the ring, after it has cooled sufficiently, place a little vaselin, while the ring is still held in sterile forceps.

3. Place the ring on the slide and press it down gently to make contact complete. The vaselin renders the chamber water tight.

4. Seal the ring to the slide with melted paraffin as in the adhesion culture, to keep it from slipping around.

5. With a sterile pipette convey into this chamber just enough (boiled) water to cover the bottom.

6. Vaseline the upper edge of the ring.

7. Inoculate lightly the tube of liquid medium with the organism to be studied. Distribute throughout the liquid with the needle.

8. Transfer one loopful to a cover-glass.

9. Using the straight needle, spread in a thin film over the entire surface of the cover-glass. If any of the liquid adheres in droplets, shake them off.

10. Press the cover-glass, medium side down, upon the upper vaselined edge of the ring.

11. Seal the edge of the cover-glass to the glass ring in several places with paraffin to prevent it from slipping around.

12. Incubate at the desired temperature.

This possesses some advantages over the adhesion culture, as more air and moisture and consequently more favorable conditions are furnished for growth. With a little more delicate manipulation agar or gelatin can be used in place of the liquid medium.

EXERCISE 24. PREPARATION OF AGAR HANGING-BLOCK CULTURE

This method was devised by Hill * for studying to better advantage the morphology and manner of multiplication of bacteria.

Carry out this procedure in a special plating room or chamber if possible, to avoid contamination from air currents.

* Hill, Journal of Medical Research, Vol. VII, March, 1902, p. 202.

Apparatus. Clean cover-glasses; clean concave slides; ordinary slides, clean; tube of sterile nutrient agar or gelatin; paraffin; two sterile Petri dishes; scalpel; platinum loop.

Culture. Pure culture of the organism to be studied.

Method. 1. Liquefy a tube of nutrient agar or gelatin, pour it into a sterile Petri dish to the depth of about 4 mm. and allow it to harden.

2. With the flame-sterilized scalpel, cut out a block of agar about 8 mm. square.

3. Raise the agar block on the blade of the scalpel and transfer it, under side down, to the center of a sterile slide.

4. With a sterile platinum loop, spread a drop of the liquid culture (or suspension of organisms from a solid culture medium) over the upper surface of the agar block as if making a cover-glass film.

5. Place the slide and block in a sterile Petri dish and incubate for ten minutes at 37° C. to dry slightly.

6. With sterile forceps, lower a clean, dry, sterile cover-glass carefully on the inoculated surface of the agar (avoiding air bubbles), so as to leave a clear margin of cover-glass overlapping the agar block.

7. Invert the preparation and, with the blade of the scalpel, remove the slide from the agar block.

8. With the platinum loop, run a drop or two of melted agar around the edges of the block. This solidifies at once and seals the block to the cover-glass.

9. Sterilize a concave slide.

10. Invert the cover-glass with the block attached on the concave slide and seal it in place, firmly, with paraffin.

11. Observe immediately and later from time to time with ocular No. 1 and objective No. 7 or the oil immersion lens.

**EXERCISE 25. LINDNER'S CONCAVE-SLIDE METHOD
FOR DEMONSTRATING FERMENTATION**

The object of this exercise is to test the fermenting power of yeasts.

Apparatus. Three clean concave slides; three clean cover-glasses; sterile filter paper (place several pieces 8 cm. square in a Petri dish and sterilize in the hot air); three tubes of sterile wort or cider; three sterile 1 c.c. pipettes; forceps; melted paraffin; platinum needles; Bunsen burner.

Cultures. *Saccharomyces cerevisiæ*; *Saccharomyces apiculatus*; *Torula rosea*.

Method. The following procedure is to be used for each organism to be tested:

1. Using the straight needle, inoculate a tube of wort with *Saccharomyces cerevisiæ* and mix well through the medium.
2. Sterilize a concave slide in the flame.
3. Using a sterile pipette, fill the concavity of the slide until the liquid "rounds up" over the concavity.
4. Holding a cover-glass in the forceps, sterilize it in the flame.
5. Lay the cover-glass on the end of the slide and push it over with the forceps until the cover-glass covers the concavity, thus sealing in the inoculated liquid. *There must be no air bubbles. The preparation must be made over again if this occurs.*
6. Remove the excess liquid with sterile filter paper, using forceps to hold the paper.
7. Seal the cover-glass with paraffin as with the adhesion culture.
8. Place the slides in a horizontal position in Petri dishes, or in a slide box as convenient.
9. Incubate at 25° to 30° for twenty-four hours. Gas bubbles will be formed in twenty-four to forty-eight hours, if any fermentation occurs.

10. Record the time of fermentation and the relative fermentation of each yeast and draw conclusions from your results.

11. Do your results coincide with those in the references given?

12. State in detail your results with any conclusions which follow from them, and point out the practical applications which may be made.

By the use of sugar broth in place of wort, this method may be employed for bacteria as well.

REFERENCES

LAFAR: Technical Mycology, Vol. II, Part 1, pp. 113, 114, and Part 2, pp. 401-407, 430-436. (Index of three volumes is in Vol. II, Part 2.)

GREEN: Soluble Ferments and Fermentation, pp. 333-362.

CONN: Bacteria, Yeasts and Molds, pp. 56-99.

EXERCISE 26. LINDNER'S DROPLET CULTURE

The object of the exercise is to isolate a single yeast cell and watch its development.

Apparatus. Sterile cover-glass (sterilize in flame); con-

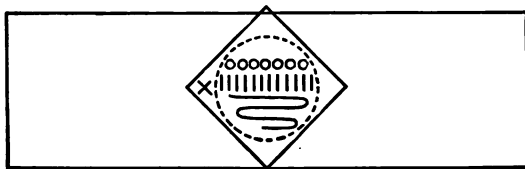


FIG. 30.—Lindner's Droplet Culture. (Adapted from Lafar's Technische Mykologie.)

cave slide; forceps; sterile toothpick (sterilize in a test tube in hot air); paraffin; India ink.

Culture. Pure culture of some yeast.

Method. 1. Inoculate a tube of cider with yeast. Distribute the organisms well.

2. Using the sterile toothpick, make five rows of small

2. Using the sterile toothpick, make five rows of small droplets (five droplets in a row) on a sterile cover-glass and place, culture side down, over the concavity of a sterile slide.

3. Seal the cover-glass with paraffin as in the preparation of the adhesion culture. Examine microscopically.

4. Locate one droplet which contains only one cell. Using India ink, write the location of this droplet on the slide.

5. Make a drawing of each stage of development until growth ceases. Why does the cell stop growing?

6. State in detail your results with any conclusions to be drawn and point out the practical applications which may be made.

This method may be used to advantage with mold spores.

EXERCISE 27. CHINESE INK PREPARATION

Chinese ink may be used to make bacteria more easily visible microscopically and to aid in taking correct measurements.

Apparatus. Sterile, dilute Chinese ink; * clean flamed glass slides.

Cultures. Pure cultures (young agar streaks are best).

Method. 1. Place one loopful of distilled water and three loopfuls of sterile Chinese ink in a row on a clean glass slide, about 2 cm. apart.

2. Inoculate the loopful of water from the original culture.

3. Distribute the organisms well with a platinum needle.

4. Then inoculate the adjoining drop of ink from the loopful of water, the second drop of ink from the first, etc.

5. Stir each loopful of ink well and then spread it so as to cover an area about 1 cm. square.

* See appendix for method of preparation.

6. Let dry. If desired the specimen may be mounted in Canada balsam before examining.

7. Examine with either the 1/7 or the oil immersion objective.

8. Write the name of the organism, the date, and your name on the glass with India ink.

By the use of the Chinese ink preparation, it is possible to examine any organism unstained. Organisms so treated neither shrink nor in any way change their form, making

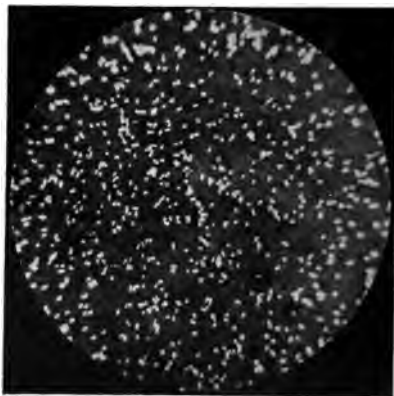


FIG. 31.—Chinese Ink Preparation. (Orig. Northrup.)

accurate measurement possible. Stains often cause organisms to appear swollen or shrunken.

The motility of bacteria may be more easily demonstrated by adding a very slight amount of this ink to a hanging drop of the organism being studied.

Caution. *Chinese ink is very expensive.* When making preparations, use every precaution to keep your supply sterile, as contaminating organisms may be confused with the culture under study. A control preparation to which no microorganisms have been added will serve to detect their presence.

EXERCISE 28. THE STAINING OF MICROORGANISMS

Microorganisms are devoid of color as a rule and are stained for the purpose of observing their morphology to better advantage than in a hanging drop. Staining also often serves to bring out certain morphological characteristics which are otherwise not evident, such as the presence of metachromatic granules or a peculiar arrangement of the protoplasm, resulting in what are known as "beaded forms."

The stains best suited to bacteria are the basic anilin dyes which are derived from the coal-tar product anilin ($C_6H_5NH_2$). Many of them have the constitution of salts.

Such compounds are divided into two groups, according as the staining action depends on the basic or the acid portion of the molecule. Fuchsin, gentian violet and methylen blue are basic dyes, while eosin, picric acid and acid fuchsin are acid dyes.

These groups have affinities for different parts of the living cells. The basic stains have a special affinity for the nuclei of tissues and for bacteria, the acid for the protoplasm and not for bacteria. The violet and the red anilin dyes in order, are the most intense in action, easily over-staining the specimen. It is difficult to overstain with methylen blue. For this reason this stain is to be preferred where the bacteria occur in thick or viscid substances, like pus, mucus or milk. In the presence of alkali, however, the stain acts more energetically.

Stock solutions of the ordinary dyes are commonly used. These are prepared by making a saturated solution of the dye in absolute alcohol; this is diluted with water as needed.

Saturated alcoholic solutions of dyes will stain bacteria with difficulty. The best results are obtained with the diluted stain, spoken of here as an "aqueous-alcoholic" stain.

Apparatus. Clean cover-glasses; clean slides; cover-glass forceps; platinum loop and needle; Bunsen burner; small pieces of filter paper; distilled water; aqueous-alcoholic solution of fuchsin, methylen blue, etc.; Canada balsam; microscope.

Note. See appendix for formulæ of stains.

- ✓ **Method.** 1. Flame a clean cover-glass, holding it by one corner with cover-glass forceps.
- ✓ 2. Place one loopful of distilled water in its center.
- ✓ 3. Touch the growth on slant agar *lightly* with a sterilized platinum needle and transfer a *very little* of the mate-

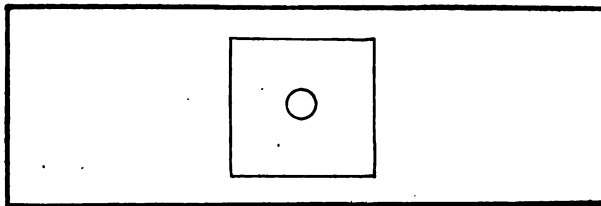


FIG. 32.—For use in mounting permanent cover-glass preparations.
(Orig.)

rial to the drop, and only sufficient to make it very slightly cloudy.

- ✓ 4. Flame the needle and allow it to cool.
- ✓ 5. Spread the drop over the entire cover-glass with one or two strokes of a straight needle. In the case of *pathogenic* microorganisms use a flat loop 2 mm. in diameter, and limit the spreading to the inner three-fourths of the cover-glass.
- ✓ 6. Allow to *dry* in air.
- ✓ 7. Fix the preparation on the cover-glass by passing the cover-glass, specimen side up, three times through the flame of a Bunsen burner. The speed is measured by moving the cover-glass and forceps in a circle of 1 ft. diameter in one second.

- ✓ 8. Flood the entire specimen-side of the cover-glass with stain, using a pipette.
- ✓ 9. Allow the stain to act a short time.

Note. The time required for staining varies so much with the different stains, different organisms and their physiological conditions, that no exact time can be given. In general, a good specimen is obtained by staining one-half to one minute with fuchsin or gentian violet, or one to five minutes with methylen blue.

- ✓ 10. Wash the specimen in running water.
- ✓ 11. Mount the cover-glass in water, specimen side down, on a clean slide.
- ✓ 12. Dry the upper surface of the cover-glass and take up any excess of water by means of filter paper.
- ✓ 13. Examine the slide under the microscope, using objective No. 7 and ocular No. 1.
- ✓ 14. If satisfactory, remove the cover-glass carefully from the slide, floating it off if necessary.
- ✓ 15. Allow it to dry in the air, specimen side up.
- 16. Place a clean slide exactly on the figure (Fig. 32).
- ✓ 17. Let a small drop of Canada balsam fall in the center of the slide, marked by the circle.

Note. The consistency of the Canada balsam should be like thin cream. The diameter of the glass rod should not be more than 4 mm.

- ✓ 18. Place the cover-glass, specimen side down, on this drop.
- 19. Allow the balsam to spread over the entire under surface of the cover-glass (without pressing it down on the slide) and keep the cover-glass straight, coinciding with the lines of the figure.
- ✓ 20. Label, stating in order, the name of the organism, the age and kind of culture, the stain used, the date, your own name and the purpose of the stain if otherwise than ordinary, e.g., spore stain.
- ✓ 21. Allow the slide to stand in a horizontal position for a few days until the balsam becomes hard.

EXERCISE 29. ANJESZKY'S METHOD OF STAINING SPORES

Spores are not stained by the ordinary staining methods, as their physical nature differs from that of the vegetative rods within which they are formed. By proper treatment with strong anilin dyes, however, it is possible to force the stain into the spore. Once within the spore it is as difficult to remove the dye as it was to cause it to enter.



FIG. 33.—Contrast Spore Stain, Carbol-fuchsin and Methylene Blue, $\times 1500$. (Orig. Northrup.)

By a careful decolorization with a weak acid, it is possible to remove the stain from everything on the cover-glass except from the spores. Then, on application of a dye of a contrasting color, the specimen will show, e.g., a bright red spore within a blue bacterium.

The fundamental principles of this method are also used for staining "acid-fast" organisms, as *Bact. tuberculosis*.

Apparatus. Clean cover-glasses; clean slides; cover-glass forceps; platinum loop and needle; Bunsen burner;

carbol-fuchsin; methylen blue, aqueous-alcoholic; hydrochloric acid, 0.5%; sulphuric acid, 5%; Canada balsam; microscope.

Culture. Culture of an organism just beginning to show spore formation.

Method. 1. Prepare a cover-glass film of the spore-containing organism and allow it to dry.

2. While it is drying, warm some 0.5% HCl in the small evaporating dish over a Bunsen burner until it steams well and bubbles begin to form.

3. When the solution is hot and the smear dry, drop the cover-glass upon the fluid and allow it to act upon the unfixed smear for three to four minutes.

4. Wash and dry the cover-glass.

5. Fix in the flame *for the first time*.

6. Stain with carbol-fuchsin by flooding the cover-glass with the stain, warming twice until fumes arise.

7. Allow to cool, and wash in water.

8. Decolorize with 5% H_2SO_4 . Spores are treated with a mild decolorizing agent, as they are much less resistant to acid than are acid-fast bacteria. (See p. 93, step 7.)

9. Wash in water.

10. Counterstain for one to two minutes with methylen blue.

11. Wash, dry and examine the specimen in water. If satisfactory, dry it and mount in balsam.

The whole procedure should not take longer than eight to ten minutes.

REFERENCE

McFARLAND: Textbook of Pathogenic Bacteriology, p. 188.

EXERCISE 30. METHOD OF STAINING TUBERCLE AND OTHER ACID-FAST BACTERIA

Acid-fast bacteria are so termed from their reaction to a special staining process. This process consists in staining the specimen containing, for example, tubercle bacteria, with hot carbol-fuchsin and decolorizing for a short time with acid; the acid takes the dye out of all other material, bacteria and blood or other body cells that may be present, leaving the tubercle bacteria stained red. This staining process is essentially the same as for spores, but the principle is different.

The property which some bacteria possess of being acid-fast is attributed to the presence of fat and wax-like substances in their cells. This seems to be proved by the fact that when the bacterial cell substance of tubercle bacteria has been freed from these fats and waxes by extraction with absolute alcohol and ether, this property is lost.

Apparatus. Clean slides; clean cover-glasses; platinum loop; copper staining dish; Bunsen burner; forceps; carbol-fuchsin; sulphuric acid, 20%; methylen blue, aqueous-alcoholic; immersion oil; Canada balsam; specimen to be examined.

Method. 1. Using a sterile loop, smear some of the specimen in the center of one surface of a clean slide, taking care not to come within 0.5 cm. of the edge.

Note. This may be applied to sputum, pus, etc. In case of tubercles or diseased organs or tissues these may be cut open with a scalpel, a portion incised, and grasping this portion with the forceps a smear made directly on the slide, following the precautions above. If pure cultures are to be examined, a cover-glass specimen may be made in the usual way.

2. Dry the slide in air.
3. Fix in the flame.
4. Support the slide on the copper staining dish; flood

the slide with carbol-fuchsin until the stain "rounds up."

5. Heat the under side of the slide directly with a flame until the carbol-fuchsin steams (*but not boils*). Keep the stain steaming for five minutes.

6. Wash in water.

7. Decolorize by dipping the slide preparation into 20% H_2SC_4 *for an instant* and washing *immediately*. This process may have to be repeated two or three times. If not careful, however, the tubercle bacteria may be decolorized. If this happens, their acid-fast property will be destroyed to some extent.

8. Counterstain with aqueous-alcoholic methylen blue.

9. Wash in water, dry and examine directly with the oil immersion lens. The specimen, if a good one, may then be mounted in the usual way without removing the immersion oil.

EXERCISE 31. METHOD FOR STAINING FLAGELLA

Flagella, the exceedingly delicate organs of locomotion of bacteria, cannot be seen in an unstained or in an ordinary stained preparation. Special staining methods must be employed to make them visible. They are generally rendered visible by precipitating some chemical on them; this generally increases their width considerably.

The staining of the flagella of bacteria is the most difficult of all bacteriological procedures and it generally requires considerable practice to insure good results.

There are many methods for staining flagella. This one, however, has met with considerable success with students. Failure to make a good flagella stain with any method is no sign that the student is not a good workman, nor is success the sign of a good bacteriologist.

Apparatus. Clean glass slides; *absolutely clean* cover-glasses; small platinum loop; several cover-glass forceps;

distilled water; mordant for flagella staining; anilin-water fuchsin or gentian violet.

Culture. Agar slant culture, twelve to eighteen hours old.

Note. The best results are obtained if successive generations of this organism have been transplanted every eighteen to twenty-four hours for several generations.

Method. 1. Place three drops of distilled water on a clean glass slide.

2. Transfer a small amount of bacterial material from

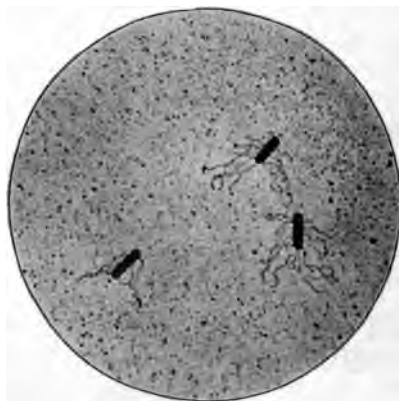


FIG. 34.—Flagella Stain. *B. typhosus*, $\times 1500$.

the moist portion of the agar slant culture to the first drop by means of a small platinum loop, using only enough of the material to make this drop very slightly cloudy.

3. Flame the needle and transfer a small portion from the first drop to the second.

4. Proceed in like manner in preparing the third dilution.

5. Place a number of *absolutely clean* cover-glasses in cover-glass forceps.

6. By means of a platinum needle bent at right angles near the end, make smears on the cover-glasses from the

second and third drops on the slide by drawing the bent needle *once, lightly* across the cover-glass. *If there is any tendency of the smear to roll or "gather in drops," the cover-glass should be discarded and a clean one substituted.* This is imperative.

7. Allow the preparation to dry for about five minutes.

8. Filter on each of the fixed smears enough of the mordant to cover the cover-glass.

9. Allow to stand about five minutes at room temperature.

10. Wash off the mordant in a small stream of water.

11. Draw off the excess water from the edge of the cover-glass by means of filter paper.

12. Stain with anilin-water fuchsin or anilin-water gentian violet for about five minutes, either cold or by warming somewhat over a low flame.

13. Wash off the excess stain with clean water.

14. Mount on a slide in water.

15. Absorb the excess water with filter paper.

16. Examine under the microscope. If the preparation has been successful, it may be dried and mounted in balsam.

EXERCISE 32. GRAM'S METHOD OF STAINING

Certain organisms, when stained with anilin-water gentian violet and afterwards treated with a solution of iodine and washed in alcohol or anilin oil, give up the stain; others retain the color when subjected to this process. These latter organisms are said to be *Gram-positive*, those losing the stain are *Gram-negative*.

This phenomenon is interpreted by Benians to be due to the possession of a definite cell-envelope which by the action of iodine is rendered impermeable to alcohol. His

experiments show that so long as the Gram-stained cell is intact, the solvent is unable to remove the stain, but that as soon as the cell is crushed and injured, the stain is, in great part, dissolved out. The amorphous débris obtained from broken-up Gram-positive bacteria does not retain Gram's stain.

Apparatus. Clean slides; clean cover-glasses; platinum loop and needle; cover-glass forceps; distilled water; anilin-water gentian violet; Lugol's iodine solution; acetone-alcohol.

Culture. Agar slant cultures preferably.

Method. 1. Prepare a cover-slip film and fix in the usual way.

2. Stain in anilin-water gentian violet three to five minutes.

3. Wash in water.

4. Treat with Lugol's iodine solution until the film is black or dark brown.

5. Wash in water.

6. Dry in air.

7. Wash in acetone-alcohol until no more color is discharged.

8. Wash in water. (Counterstain at this point if desired.)

9. Dry in air.

10. Mount in Canada balsam.

Note. The Gram-Weigert method is more applicable in case of sections of tissues. The directions from 1-6 are the same. The specimen is washed in anilin oil 1 part, xylol 2 parts, instead of alcohol, washed further in xylol and mounted at once in Canada balsam. *Bact. bulgaricum* in milk is very beautifully demonstrated by this modified method.

A few of the more common Gram-positive and -negative organisms are appended. This is not as important a diagnostic method as has been formerly supposed, because the reaction occurring often depends upon the age of the culture, the medium on which it is grown, etc.

GRAM-POSITIVE ORGANISMS. GRAM-NEGATIVE ORGANISMS.

<i>Staph. pyogenes aureus</i> and <i>albus</i>	<i>Bact. mallei</i>
<i>Strept. pyogenes</i>	<i>Bact. aerogenes</i>
<i>Bact. anthracis</i>	<i>B. typhosus</i>
<i>Bact. tuberculosis</i>	<i>B. coli communis</i>
<i>B. alvei</i>	<i>B. cholerae suis</i>
<i>B. tetani</i>	<i>M. gonorrhoea</i>
<i>Bact. acidi lactici</i>	<i>Msp. denekae</i>
<i>Bact. bulgaricum</i>	<i>Msp. finkler-prior</i>
<i>B. megaterium</i>	<i>Spirocheta obermeieri</i>
<i>B. subtilis</i>	<i>Proteus vulgaris</i>
<i>B. mycoides</i>	<i>Ps. medicaginis</i>
<i>B. mesentericus vulgatus</i>	<i>B. amylovorus</i>
<i>M. tetragenus</i>	<i>Ps. campestris</i>
<i>Streptothrix actinomyces</i>	<i>B. phytophthorus</i>
<i>Sacch. cerevisiae</i> and other yeasts	<i>B. caratovorvus</i>
Molds	

REFERENCE

- BENIANS, T. H. C.: "Observations on the Gram-positive and Acid-fast Properties of Bacteria." Jour. of Path. and Bact., Vol. XVII, pp. 199-211 (1912). ✓

EXERCISE 33. METHOD FOR STAINING CAPSULES

Some bacteria possess a gelatinous envelope or "capsule" which in some species surrounds each individual organism, and in others, groups of organisms. The presence of this capsule may be demonstrated by various special staining methods. The capsule takes the stain much less quickly than does the organism, leaving a light-colored halo about it. The presence of a capsule does not always indicate that the organism forming it is a slime-forming organism, nor does the fact that an organism is a slime-former preclude the possession of a capsule.

Apparatus. Clean cover-glasses; clean slides; platinum loop; cover-glass forceps; filter paper, pieces; glacial acetic acid; gentian violet, aqueous-alcoholic.

Cultures. Cultures in milk, serum, etc., media.

Method. 1. Prepare the cover-glass specimen directly from the medium *without the use of water*. Spread and fix in the usual manner.

2. Flood the specimen side of cover-glass with *glacial* acetic acid.

3. Drain immediately without washing. A piece of filter paper may be touched to the edge of glass to take up surplus water and facilitate drainage.

4. Stain with aqueous-alcoholic gentian violet for a few seconds.

5. Examine under the microscope.

6. Wash, dry and mount.

EXERCISE 34. METHOD OF MAKING IMPRESSION PREPARATIONS

Impression preparations (Klättschpräparat) are prepared from isolated colonies of bacteria in order that their characteristic formation may be examined by higher powers than can be used with the living cultivation *in situ*. They are prepared from plate cultivations. Young colonies of *Bact. anthracis* produce beautiful impression preparations.

Apparatus. Clean cover-glasses; clean slides; Novy cover-glass forceps; dissecting needle; stain.

Culture. Agar plate culture containing well-isolated colonies of organism to be studied.

Method. 1. Taking a clean cover-glass in the Novy forceps, open the plate and rest one edge of the cover-glass on the surface of the medium a little to one side of the selected colony.

2. Lower it carefully over the colony until horizontal. *Avoid any lateral movement or the inclusion of air bubbles.*

3. Press gently on the center of the upper surface of the cover-glass with the points of the forceps to insure perfect contact with the colony.

4. Steady one edge of the cover-glass with the forceps and pass the point of the dissecting needle just under the opposite edge and raise carefully; the colony will be adherent to it.

When nearly vertical, grasp the cover-glass with the forceps and remove it from the plate. Re-cover the plate.

5. Place the cover-glass specimen side up on desk and cover with half a Petri dish until dry.

6. Fix in the flame.

7. Stain and mount as with ordinary cover-glass specimen, being careful to perform all washing operations with extreme gentleness.

EXERCISE 35. METHOD OF STAINING THE NUCLEI OF YEAST CELLS

The nuclei of yeast cells are not visible in unstained or in ordinary stained specimens. A special method of procedure must be used.

Apparatus. Clean cover-glasses; clean slides; forceps; ferric ammonium sulphate, 3% aqueous solution; Ehrlich's hematoxylin solution; two staining dishes for slides.

Culture. Culture of *Saccharomyces* or *Torula*.

Method. 1. Prepare and fix the film upon the slide in the usual way.

2. Soak in 3% ferric ammonium sulphate for two hours.

3. Wash thoroughly in water.

4. Stain in hematoxylin solution for thirty minutes.

5. Wash in water.

6. Differentiate in ferric ammonium sulphate solution for one and a half to two minutes, examining wet under the microscope during the process.

7. Wash, dry and mount.

GENERAL CHARACTERISTICS OF MOLD GROWTH AND HINTS FOR STUDY

A brief description of the molds to be studied in the laboratory is here given. The references cited will give more in detail of their structure, importance and occurrence.

In these descriptions, there have been noted the parts of the structure of each mold that are to be found microscopically and drawn, also the quickest method of obtaining the best results. All microscopic drawings and measurements can be secured from the adhesion culture or the moist-chamber culture.

Rhizopus nigricans—Black mold

(*Mucor stolonifer*)

The mycelium in the advanced stage consists of rhizoids (rootlets), bearing clusters of sporangiophores, joined by long hyphæ (the stolons) to the mycelium proper. The hyphæ are non-septate.

The fruiting bodies consist of typical sporangia (spore cases containing spores) borne on the enlarged end (columella) of the sporangiophore. Spores are liberated by the bursting of the sporangium.

The columella can be observed in fruiting bodies of a light brown color; white sporangia are too young and black too old to show this structure. If no fruiting bodies grow in the adhesion culture, they may be studied directly from a plate culture by preparing a glycerin slide. Take care not to burst the sporangium when transferring it to the slide.

Aspergillus niger—Black mold

The mycelium of this mold consists of septate hyphæ with frequent dichotomous branching.

The fruiting body (asexual) consists of an erect conidiophore usually ending more or less abruptly in a dilation or head which bears closely packed sterigmata each of which in turn bears a single chain of conidia, the newly formed conidium being pushed away by the formation of a new spore; thus the conidium at the end of the chain is the oldest. The conidia of this mold are black.

Penicillium italicum—Blue-green mold

The mycelium consists of septate hyphæ, having frequent dichotomous branching.

The conidial fructification resembles a brush, the conidia (spores) being borne on the end of conidiiferous cells (sterigmata); in this genus before the conidia appear, there is generally a primary and even a secondary branching of the conidiophore in some species before the conidiiferous cells are formed. The species of *Penicillium* have more of a brush-like appearance than the species of *Aspergillus*. The spores of *P. italicum* are blue-green.

Oospora (Oidium) lactis—White mold

The mycelium consists of septate hyphæ, having dichotomous branching; the hyphæ are almost entirely submerged in the nutrient substrate.

This differs from the other molds in that it does not have typical fruiting bodies. It reproduces by means of conidia, which are formed by a simple division of the hyphæ. The conidia are colorless.

REFERENCES

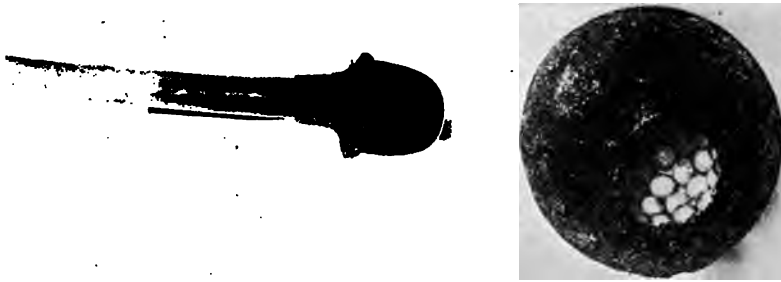
- MARSHALL: Microbiology, pp. 12-27.
LAFAR: Technical Mycology, Vol. II, Part I, pp. 5, 15, 71-77; Part II, pp. 300-346, 451-455.
KLÖCKER: Fermentation Studies, pp. 184, 185, 274-282, 303, 304.



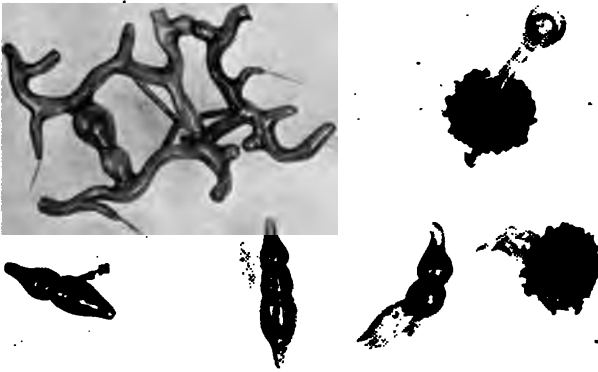
Germination of *Rhizopus* Spore, Mycelium, Rhizoids and Development of Sporangiohores and Sporangium.



Ripe Sporangium.



Sporangiophore with Columella Attached and Ripe Sporangium Showing Spores Within.



Various Stages in the Formation and Germination of a Zygospore.

* Plates I-VI photographed from models manufactured by R. Brendel, Berlin—Grunewald, Bismarck—Allee 37, Germany.



EXERCISE 36. MICROSCOPICAL EXAMINATION OF MOLDS

Apparatus. Clean cover-glasses; clean slides; hand lens or compound microscope; platinum needle and loop; dissecting needle; glycerin, 10%.

Cultures. Plate culture of mold.

Method. The gross structure of a mold colony upon a plate may be examined with a hand lens or by placing the inverted Petri dish culture on the stage of the compound microscope and examining with objective No. 3 and ocular No. 1. The structure may be examined in detail as follows:

1. Select a young colony which shows colored fruiting bodies, if such are produced by the organism to be studied. (Growth from natural or artificial media may be treated in the same general way.)

2. Using a sterile platinum needle, transfer a small portion of the mycelium and fruiting bodies to a drop of glycerin on a plain glass slide. If the mold growth is closely confined to the surface of the media (as with *Penicillium* or *Aspergillus*), it is often desirable to cut out a small piece of the medium bearing the mold and lift to the slide by means of a sterile platinum loop.

3. Tease out very gently, using dissecting needles or common pins. The mold structure is extremely delicate, so this operation must be performed with the utmost care.

4. Place a cover-glass over the preparation.

5. Examine with the microscope, using objective No. 3 and ocular No. 1. When a portion of mycelium bearing fruiting bodies is found, examine with objective No. 7. Draw the young and the old fruiting organs.

DESCRIPTION OF PLATE III

Aspergillus, Showing Septate Mycelium, Conidiophore with Conidia, also Formation of Asogonium.

Penicillium, Germination of Spore, Formation of Mycelium, Septate Conidiophores with Conidia, Ripe Ascospore.



EXERCISE 37. THE STUDY OF MOLDS

Apparatus. Ten sterile Petri dishes; four tubes of sterile slanted agar; ten tubes of sterile agar, for plates; four tubes of sterile cider or wort; four tubes of sterile gelatin; clean glass rings, slides and cover-glasses; hand lens; compound microscope; centimeter scale.

Cultures. Pure or mixed cultures of the following four molds: *Rhizopus nigricans*; *Aspergillus niger*; *Penicillium italicum*; *Oospora lactis*. Mixed (or impure) cultures of two molds growing in their natural habitat will be found on each table.

Method. 1. Plate out each mixed culture* making three straight needle dilution plates for each. Use agar as a medium. Place the plates in the constant-temperature room in the place assigned. Note the temperature.

2. When the plates are twenty-four hours old, mark and draw a well-isolated typical colony of the mold from the most thinly populated plate. Measure and record the diameter of the colony in millimeters.

3. When fruiting bodies begin to show, isolate a pure culture of each mold in cider (see Exercise 16).

4. (a) As soon as growth begins to show in the tubes of cider (about twenty-four to thirty-six hours) make a macroscopic drawing of each. State the age of the culture.

(b) When mycelium is well developed and fruiting bodies appear (as noted on plates) make a second drawing.

*Two mixed and two pure cultures are furnished for study. These cultures owe their color to the presence of fruiting bodies or spores. Always endeavor to obtain spores when making inoculations from molds.

DESCRIPTION OF PLATE IV

Sexual Reproduction of *Penicillium*, Ascus Formation.

Formation of Chains of Asci in Process of Ripening, Some Containing Ascospores, Section of a Ripe Ascus.

Saccharomyces, Budding, Colony Formation, Production of Ascus and Germination of Ascospores.

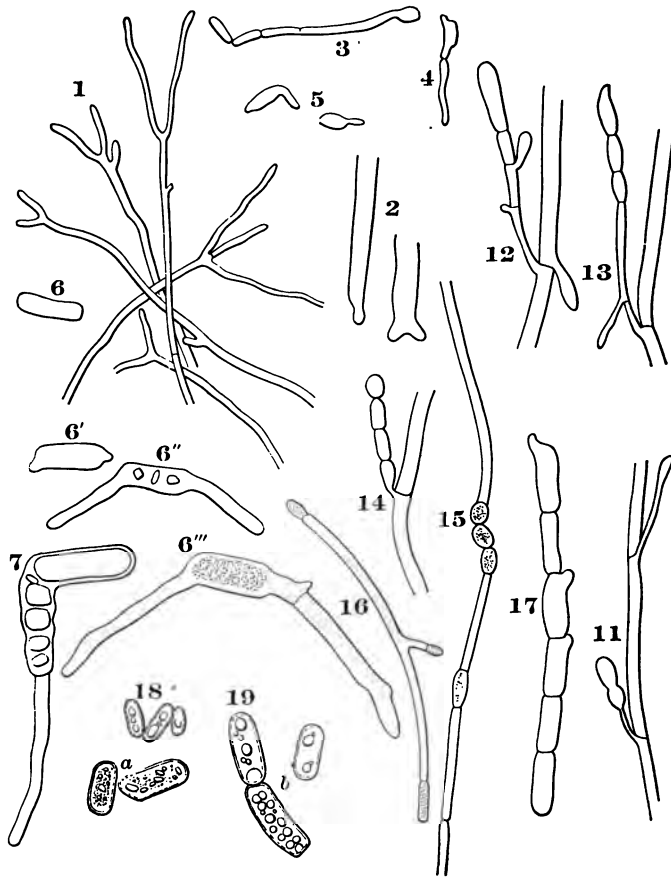


FIG. 35.—*Oospora (Oidium) lactis* (after Hansen) see p. 104, Jörgensen.

1. Hyphæ with forked partitions; 2, two ends of hyphæ—one with forked partition, the other with commencement of development of a spherical link; 3-7, germinating conidia; 6-6''', germination of a conidium, sown in hopped beer-wort in Ranvier's chamber, and represented at several stages; at each end germ tubes have developed; after nine hours (6''') these have formed transverse septa and the first indications of branchings; 11-14, abnormal forms; 15, 16, hyphæ with interstitial cells, filled with plasma; 17, chain of germinating conidia; 18, conidia which have lain for some time in a sugar-solution; the contents show globules of oil; 19, old conidia.

State the age of the culture. These cider tubes are then of the proper age from which to make inoculations.

5. *Pure cultures* of the two remaining molds will be found in tumblers marked "Laboratory Cultures."

Always leave such cultures in the place assigned, after using.

Make cultures of each of the four molds as follows:

(a) Agar slant (for method see Exercise 15).

(b) Agar plate (giant colony). Use Roux culture flask for *Rhizopus nigricans* (see pp. 2, 62).

(c) Cider or wort (test-tube culture).* For method of inoculation of *c* and *d* see pp. 60, 61.

(d) Gelatin stab (test-tube culture). Keep all gelatin test-tube cultures *in cold-water bath* or in a cool place (15° to 20° C).

(e) Adhesion or moist-chamber culture. (See pp. 78-81). Prepare these cultures from the *freshly inoculated* cider tubes.

6. *Make drawing of spores of each mold* from adhesion or moist-chamber culture *as soon as preparation is made*. Measure the spores and record the limits of size.

7. Draw the twenty-four hour cultures each of *a*, *b*, *c*, *d*, and *e* and label in detail. Measure the spores which have germinated in *e*, and record the diameter and length of the mycelium.

8. Make drawings of branched mycelium and several stages of development of fruiting bodies from a glycerin mount. This mount is most easily prepared from an agar plate colony.

9. Make drawings of all cultures as soon as a *marked* development is seen over that of the preceding drawing. Three drawings of each culture should be sufficient.

10. Measure giant colony of each mold every day and record the measurements. What is noted of the comparative rate of growth?

All drawings must be made directly on charts or in note-

* Cider cultures have already been made of the two molds isolated from mixed culture.





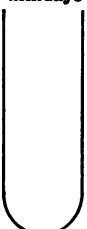



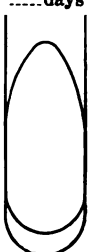
books as assigned. *Describe the drawings at the time they are made.* Descriptions are to be recorded in ink; use a 6H pencil for drawings.

This outline or some modification of it may be employed for the various species of molds.

MOLDS

Name of student	Desk No.
Name of organism	Isolated from
Method of isolation	
Occurrence	
Importance	
Spore	
Stages of germination	Drawn from preparation
Mycelium	Drawn from preparation
Method of reproduction	Drawn from preparation
Total organism	Drawn from preparation

Note: Mold, yeast and bacteria charts (pp. 110-111, 122-123, 135-138) may be procured from the College Printery, East Lansing, Mich.

	1 daydaysdays
Cider or wort culture			
Titre.....			
Incubated atC.			
Nutrient gelatin stab			
Titre.....			
Incubated atC.			
Agar streak			
Titre.....			
Incubated at° C.			
Age of colony			
Size of colony			
Surface elevation			
Gelatin or agar colony			
Titre.....			
Incubated at° C.			

EXERCISE 38. TO DETERMINE THE ACIDITY CHANGES PRODUCED BY MOLDS IN CIDER (OR OTHER LIQUID MEDIA HAVING A LOW ACIDITY AND LOW SUGAR CONTENT)

Apparatus. Four 100 c.c. Erlenmeyer flasks; 200 c.c. of cider; six 1-c.c. pipettes for titration (sterile); normal NaOH; N/20 NaOH.

Cultures. Pure cultures of four molds.

Method. 1. Determine the titre (reaction) of the cider and neutralize with normal sodium hydrate.

2. Place 50 c.c. in each of the Erlenmeyer flasks and sterilize by the Tyndall method.

3. Inoculate each, using a different mold for each flask.

4. As soon as the mold mycelium shows in the flask (examine by looking through the flask toward the light), titrate.

Note. One cubic centimeter of neutral cider is diluted to 50 c.c. with distilled water for titrating as the larger quantity, 5 c.c., when diluted, is of such a dark color that it is practically impossible to obtain a uniform or satisfactory end point. The burette reading must be multiplied by 5.

Each pipette must be used *only once*. After using a pipette once, clean and resterilize it for future use.

5. Titrate every three days thereafter, making eight titrations in all.

6. Tabulate your results. Plot a curve showing the rise in acidity, making all curves on one sheet, starting from the same zero point, and using different inks or different kinds of lines to represent the different acidity curves. Use acidity values as ordinates, days as abscissæ.

7. State fully any conclusions which may be based upon your data and point out the practical application which may be made.

REFERENCE

LAFAR: Technical Mycology, Vol. II, Part II, pp. 353-361.

EXERCISE 39. TO DEMONSTRATE THE PATHOGENIC NATURE OF MOLDS

Apparatus. One deep culture dish; one perfect fruit the same as that from which the mold was isolated, or any fruit which is the natural habitat of the mold.

Cultures. Pure culture of a mold isolated from a fruit.

Method. 1. Make small circles on opposite sides of the fruit with the wax pencil.

2. Puncture the center of one circle with a sterile platinum needle.

3. Then with needle contaminated with the mold spores inoculate the circle on the opposite side by puncturing as in 2.

4. Place at about 25° C. and observe from day to day for two weeks. Cut the fruit through the points of inoculation and note results.

5. How do fruits usually become contaminated with molds? What preventive measures would you suggest?

6. What is a *perfect* fruit from the bacteriological standpoint? From the horticultural standpoint? May these view-points differ? If so, how? What other fruits would be susceptible theoretically to the mold you used? Why?

What other types of microorganisms may be pathogenic to fruits?

7. State in full the results obtained, with any conclusions that may be drawn, and point out the practical application which may be made.

REFERENCES

MARSHALL: Microbiology, p. 513.

SMITH, ERWIN F.: Bacteria in Relation to Plant Diseases, Vol. I, p. 202, Plates 29, 30 and 31.

SMITH, ERWIN F.: Bacteria in Relation to Plant Diseases, Vol. II, Fig. 13, pp. 60 and 174-181.

YEASTS

The so-called yeasts are divided into true yeasts "*Saccharomycetes*" (wild and cultivated), and pseudo-yeasts or false yeasts, "*Torulæ*" and "*Mycodermata*."

By true yeasts are meant those which usually produce alcoholic fermentation (*Sacch. membranæfaciens* is an exception), and which are able to form endospores.

Pseudo-yeasts do not form endospores and produce little or no alcoholic fermentation.

Sacch. cerevisiæ, the yeast used in the manufacture of beers and in bread-making, is a good example of the cultivated yeast.

Sacch. apiculatus and *Sacch. ellipsoideus* are examples of yeasts which are involved in the making of wines. (The latter is cultivated and pure cultures used to some extent.)

Torula rosea is an example of the pseudo-yeast. These look like true yeasts, reproduce by budding, but seldom produce alcoholic fermentation.

REFERENCES

MARSHALL: Microbiology, pp. 28-36, 420-423, 440, 460.

KLÖCKER: Fermentation Studies, pp. 205, 249, 289, 296.

EXERCISE 40. TO ISOLATE A PURE CULTURE OF SACCHAROMYCES CEREVISIÆ AND TO STUDY THE FLORA OF A COMPRESSED YEAST CAKE

Apparatus. Cover-glasses; concave slide; sterile Esmarch dishes; potato knife; platinum needles; Bunsen burner; sterile pipette; three tubes of sterile dextrose agar; iodine solution; methylene blue (0.0001% aqueous solution); three sterile Petri dishes.

Culture. Fresh compressed yeast cake.

A. Isolation of *Saccharomyces Cerevisiæ*

Method. 1. Sterilize the potato knife in the flame of the Bunsen burner.

2. As soon as cool, cut a piece off the yeast cake.

3. Make three dilution plates in dextrose agar immediately from this freshly cut surface. *Use the straight needle* and transfer only a *very minute* quantity of the yeast. Distribute well with the platinum needle. Use the *straight needle* for making dilutions *in all cases*.

4. When the colony develops (three to six days) examine under objective No. 3, ocular No. 1, inverting the plate for this purpose.

The individual cells of most yeast colonies may be seen under objective No. 3, while individual bacteria can seldom be distinguished in the colony at this low magnification.

5. When you have located a yeast colony make a hanging drop from it in water and determine the shape of the individual yeast cells.

6. If they have the shape and size of *Sacch. cerevisiæ* (see Marshall, p. 32), inoculate a tube of wort from this colony.

7. Study this yeast according to directions in Exercise 42.

B. Study of Flora of Compressed Yeast Cake

Method. 1. After preparing plates, place the yeast cake in a sterile Esmarch dish.

2. Add 1 c.c. of boiled water, using a sterile pipette.

3. From the freshly cut surface, prepare a hanging drop of the yeast in water, adding a loopful of iodine solution to it. Yeast cells will be unstained, while starch grains become blue.

4. Repeat every seven days.

5. Is the cake made up mostly of starch grains or yeast cells? What is the purpose of the starch in the yeast cake? Do the starch grains remain intact or do they disappear? Explain. What kinds of starch are used?

6. Draw and measure the starch grains. A drawing of the individual yeast cell may be made from this mount.

7. Prepare a second hanging drop of yeast in water from the fresh cake.

8. Stain by adding a loopful of 0.0001% aqueous methylen blue. Dead yeast cells are stained blue, while the living cells remain unstained.

9. Count the number of living and dead cells in each of several fields. Estimate the per cent of living and dead yeast cells.

10. Repeat every seven days until all the yeast cells are dead.

11. How long does this take? What factors influence the death rate? Do other microorganisms enter? If so, what types? Why? From what source? Do they influence the value of the yeast cake? How?

12. Each time you record the percentage of living and dead cells, note the macroscopical appearance of the cake. Also note the presence of new microorganisms, consistency of the cake, odor, and color.

13. Record the results of this experiment in tabulated form, and state any conclusions that may be drawn or practical application to be made.

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- CONN: *Yeasts, Molds and Bacteria*, pp. 56-99.
- SCHNEIDER: *Bacteriological Methods in Food and Drugs Laboratories*. (1915). Plate I, Figs. 2, 3 and 4.

EXERCISE 41. APPARATUS AND METHODS FOR THE STUDY OF GASEOUS FERMENTATION

Various forms of yeasts, bacteria and other microorganisms have the ability to ferment carbohydrate, nitrogenous, and other food substances with the liberation of gas.

A. Smith's Fermentation Tube

Theobald Smith (1893) introduced the use of a special tube for studying fermentation and gas production, and now Smith's fermentation tube is in general use in this and other countries.

Its value lies in the fact that it is a simple apparatus, yet it allows not only of testing the relative fermentative powers of different species of microorganisms or of different strains of the same species, but of determining the gases produced qualitatively and their relative proportions quantitatively to some extent.

Apparatus. Smith fermentation tubes; gasometer; nutrient carbohydrate broth (or any desired solution); platinum needles.

Culture. Culture of the organism to be tested.

Method. 1. The carbohydrate broth (or other liquid medium) is placed in the fermentation tubes, filling the long arm by carefully tilting. The bulb should be filled with the liquid only to the extent that air will not enter the long arm upon slightly tilting. The tube should not be filled so full that the bulb will not contain all of the liquid in the long arm.

2. Sterilize. Carbohydrate broths are sterilized by the intermittent method.

3. Inoculate fermentation tubes of the desired medium with the organism to be tested, using a loop or straight needle.

4. Incubate at optimum temperature.

5. Examine in twenty-four hours for gas production, and mark the level of the liquid in the long arm of the

fermentation tube each day if gas is being formed. (If the level is *higher* than it was the previous day, the gas (CO₂) is being *absorbed*. Do not allow this absorption to proceed further, but test the gas present for CO₂ and H₂).

6. Measure and record the amount of gas by means of a gasometer (see illustration). The total amount is not

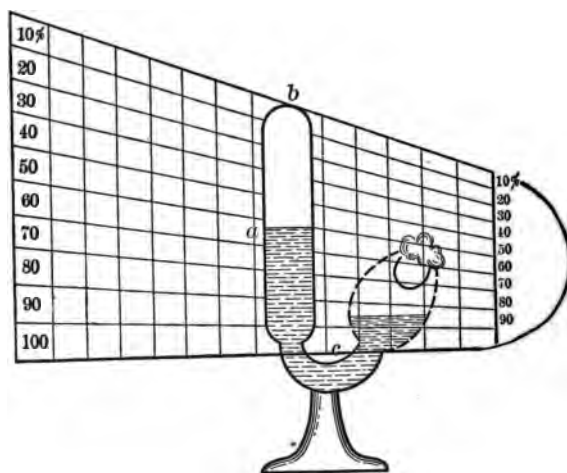
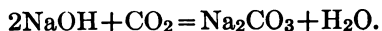


FIG. 36.—Smith's Fermentation Tube Showing use of Frost's Gasometer.

exact quantitatively, as some gas is given off from the open arm of the fermentation tube.

7. When the maximum amount of gas is formed, test the gas for CO₂ and other gases as follows:

Fill the short arm of the fermentation tube with 10% NaOH. Place the thumb over the mouth of the tube and shake vigorously, so that the gas contained in the long arm comes in contact with the NaOH.



Sodium carbonate and water are formed, leaving the other gases free.

Collect in the long arm of the tube all the gases remaining. Remove the thumb. The difference in the per cent of gas before and after treating with the NaOH equals the per cent of CO₂ which was present.

8. Place the thumb over the mouth of the tube and *collect all remaining gas in the short arm.* Light a match, remove thumb and *immediately* touch off the remaining gas. If H₂ is present the typical reaction occurs. Other gases are often present, but in too small amounts to allow of testing.

10. Record the relative proportions of CO₂ and H₂ formed.

B. Durham's Fermentation Tube

Durham's fermentation tube is simply an ordinary test tube containing a sugar broth, in which a smaller test tube, inverted, has been placed before sterilization.

This apparatus possesses some advantages over the Smith fermentation tube if only the presence of gas production is to be noted, as the tubes are more easily cleaned, sterilized and handled.

The amount of gas may be roughly estimated, but the kind of gas may not be determined by the use of this apparatus.

EXERCISE 42. THE STUDY OF YEASTS

The object of this exercise is to demonstrate how to differentiate yeasts by microscopical and cultural methods.

Apparatus. Clean cover-glasses; three clean concave slides; five clean fermentation tubes; one tube sterile 2% dextrose broth;* one tube sterile 2% lactose broth;

* The sugar and glycerin broths are furnished by the laboratory.

2% dextrose broth; one tube sterile 2% lactose broth; one tube sterile 2% saccharose broth; one tube sterile 2% glycerin broth; one tube sterile nutrient broth; three tubes sterile wort; three tubes sterile gelatin; four tubes sterile dextrose agar; gasometer; 10% NaOH; litmus solution.

Cultures. *Saccharomyces cerevisiae*;* *Saccharomyces apiculatus*; *Torula rosea*.

Method. 1. Fill one fermentation tube with each broth. Sterilize by heating one-half hour in the steam on three consecutive days.

2. Make cultures of each yeast in

- (a) Beer wort.
- (b) Gelatin (stab culture).
- (c) Dextrose agar slant.
- (d) Dextrose agar plate (giant colony).†
- (e) Lindner's concave slide culture (p. 83).

3. Make cultures of *Saccharomyces cerevisiae* only, in fermentation tubes of

- (a) Plain broth—control—(without carbohydrate).
- (b) 2% dextrose broth.
- (c) 2% lactose broth.
- (d) 2% saccharose broth.
- (e) 2% glycerin broth.

4. Prepare an *adhesion* culture from freshly inoculated wort culture of the yeast (see p. 78).

5. Examine microscopically immediately after preparation and draw single cells and cells in various stages of budding (gemination); show interior structure of cells.

6. Examine all cultures after twenty-four hours, and make drawings of (a), (b), (c), (d), and (e) under 2, place as indicated on chart, and label correctly.

7. Describe all gelatin and agar cultures according to the descriptive chart of the American Society of Bacteriol-

* *Saccharomyces cerevisiae* has been previously isolated from a fresh cake of Fleischmann's compressed yeast. (See Exercise 40.)

† Only one plate is necessary. All yeasts may be grown on one plate. Use dextrose agar.

ogists (p. 134). In describing the wort culture use the descriptive chart terms under the heading "Nutrient broth."

8. If any gas has formed in the fermentation tubes mark the level of the liquid in the long arm with a wax pencil and record the percentage of gas, using the gasometer. Test for acid, and alcohol (odor).

9. Test quantitatively and qualitatively for gas in the fermentation tubes. (See Exercise 41, p. 117).

10. What is the ratio of the CO_2 to H_2 and other gases? Is this ratio constant for all fermentations? For one organism? Why? Do all organisms cause fermentation? Why? What causes fermentation?

11. Examine adhesion cultures after forty-eight hours and seventy-two hours and make drawing of colony formation.

12. Study the fourteen to twenty-day old wort cultures in hanging drop for endospores. When do these form? Why?

REFERENCES

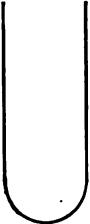
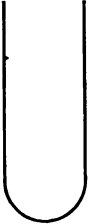

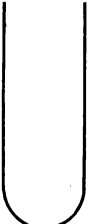
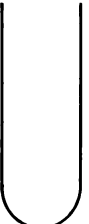




- HANSEN, E.: Practical Studies in Fermentation, pp. 215-217.
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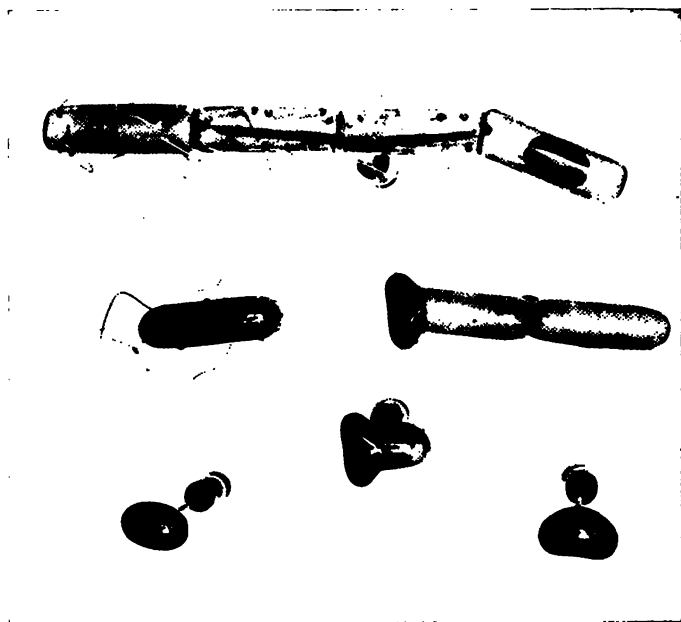
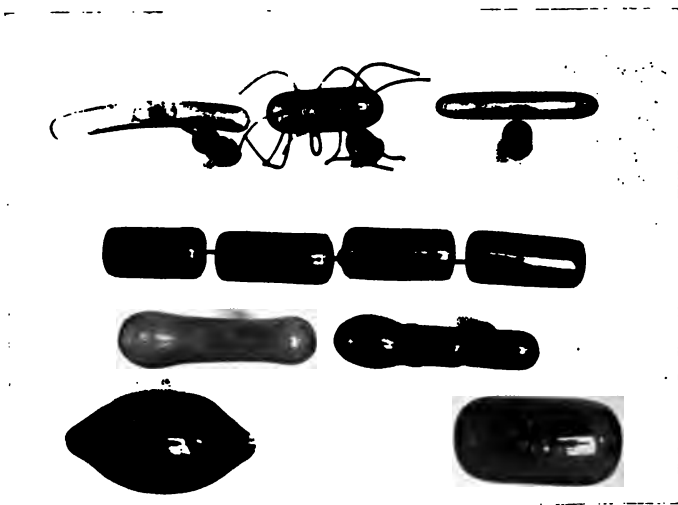
YEASTS

Name of student	Desk No.
Name of organism	Isolated from
Method of isolation	
Occurrence	
Importance	
Total organism	Drawn from.....preparation
Stages of budding	Drawn from.....preparation
Method of reproduction	Drawn from.....preparation
Spore	

Fermentations

% Of gas in	Control	Dextrose	Lactose	Saccharose	Glycerin
24 hours					
48 hours					
3 days					
5 days					
Total gas production					
Ratio of CO ₂ :H ₂ and other gases					
Alcohol (odor)					
Acid					
Growth in closed arm					

	1 daydaysdays
Cider or wort culture Titre..... Incubated at° C.			
Nutrient gelatin stab Titre..... Incubated at° C.			
Agar streak Titre..... Incubated at° C.			
Age of colony			
Size of colony			
Surface elevation			
Gelatin or agar colony Titre..... Incubated at° C.			



EXERCISE 43. THE STUDY OF BACTERIA

Studies will be made of ten bacteria representing the different morphological types. These are to be identified by morphological and cultural characteristics.

Pure cultures of these organisms will be found on each desk in the tumblers marked "Laboratory cultures." Always return laboratory cultures to these tumblers immediately after using.

DANGER. Some of these organisms are pathogenic. If you do not handle them with care and according to directions you endanger not only yourself, but all working in the laboratory. Do not be careless. Handle all organisms as if they were pathogenic. This is a good habit; get it immediately. (See "Care of Cultures," pp. 46-48) The instructor will designate which organisms are pathogenic.

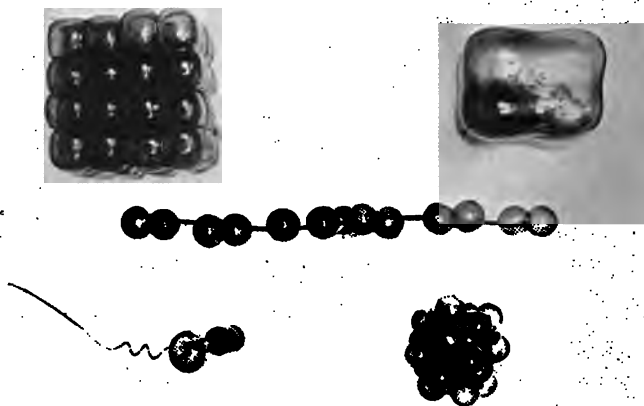
Apparatus. Clean cover-glasses; clean concave slides; clean plain slides; ten agar slants; ten tubes sterile agar for plates; ten tubes nutrient broth; ten tubes nutrient gelatin; ten tubes litmus milk; ten tubes glycerin potato; ten tubes Dunham's solution; ten tubes nitrate peptone solution; four fermentation tubes of plain broth; four fermentation tubes of dextrose broth; four fermentation tubes of lactose broth; four fermentation tubes of saccharose broth; centimeter scale; gasometer; lead acetate

DESCRIPTION OF PLATE V

- I. 1, *Bact. tuberculosis*; 2, *B. typhosus*; 3, *Bact. lepræ*; 4, *Bact. anthracis* (strepto-bacterium, two with spores); 5, *Bact. diphtheriæ* (club-shaped); 6, anthrax spore, germinating (polar); 7, *B. amylobacter* (clostridium); 8, *Streptococcus pneumoniae* (diplococcus with capsule).
- II. 1, *B. subtilis* (strepto-bacillus, peritrichous flagella, one with spore); 2, *B. subtilis* (peritrichous flagella); 3, formation of a new filament from a germinating spore; 4, spore of *B. subtilis*; 5, germinating spore of *B. subtilis* (equatorial); 6, beginning germination.



III. 1, *Spirillum volutans* (Cohn) with lophotrichous flagella (chain of three); 2, *Sp. volutans*, single cell; 3, *Microspira comma*, monotrichous flagellum; 4, *Spirocheta obermeieri*.



IV. 1, *Sarcina lutea*; 2, *Micrococcus tetragenus* with capsule; 3, streptococcus; 4, planococcus; 5, staphylococcus.

paper; aqueous-alcoholic fuchsin and methylen blue; mordant for flagella stain; Lugol's iodine solution; anilin-

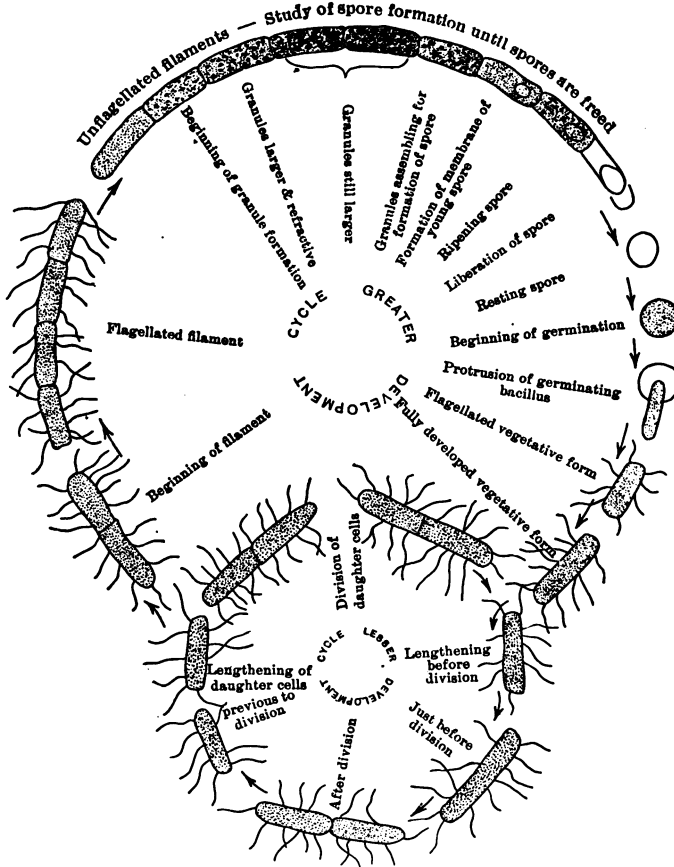


FIG. 37.—Cycle of Development of Bacterial Cell. (Adapted from Fuhrmann's Technische Mykologie.)

water gentian violet; carbol-fuchsin; acetic acid-alcohol for decolorizing spore stain; indol test solutions; nitrate test solutions; ammonia test solutions.

Method. 1. Make an agar slant culture of each organism and incubate each at its optimum temperature. (Instructor will designate the optimum temperature of each.)

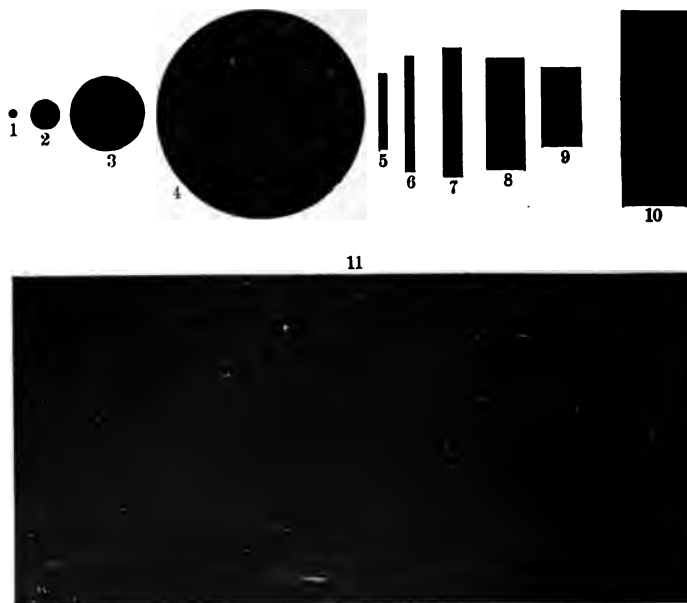


FIG. 38.—Comparative Sizes of Bacteria.

- 1, *Micrococcus progrediens*, 0.15μ ; 2, *Micrococcus urea*, $1-1.5\mu$;
- 3, *Sarcina maxima*, 4μ ; 4, *Thiophysa volutans* (sulphur bacteria), $7-18\mu$;
- 5, influenza bacillus, $4.2 \times 0.4\mu$; 6, methane bacillus, $5 \times 0.4\mu$;
- 7, *Urobacillus duclauxii* (Miquel), $2-10 \times 0.6-0.8\mu$;
- 8, *Bacillus nitri* (Ambroz), $3-8 \times 2-3\mu$; 9, *Beggiatoa alba* (sulphur bacteria), $2.9-5.8 \times 2.8-2.9\mu$;
- 10, *Chromatium okenii*, (sulphur bacteria), $10-15 \times 5\mu$;
- 11, *Beggiatoa mirabilis* (sulphur bacteria), $20-25 \times 40-50\mu$. (From Fuhrmann's Technische Mykologie.)

2. Draw and describe twenty-four-hour old agar slant cultures, then examine microscopically in hanging drop to determine the morphology, size, grouping or arrangement,

motility, spores. Use ocular No. 1 and objective No. 7. The greatest motility will be observed in organisms growing in the condensation water at the base of the slant.

3. Draw the total organism and record the presence or absence of motility. *Describe all cultures at the time the drawings are made* of each, following the terminology of the "Descriptive Chart of the American Society of Bacteriologists," p. 134.

4. Use drawing pencil for making drawings and ink for recording descriptions.

Any descriptive terms may be added which will aid in identifying organisms, but descriptive chart terms must be followed as closely as possible, otherwise *drawings will not be accepted*.

Always state the *age of the culture*, the *temperature at which the organism is grown*, the *medium* upon which it is cultivated and the *titre of the medium*.

Use one chart for each organism.

5. When the agar slant culture of each organism shows good growth, make inoculations from this culture into the following media:

Agar plate (see below for method).

Gelatin plate (see step 6, below).

Nutrient broth.

Nutrient gelatin (stab culture).

Litmus milk.

Glycerin potato.

Dunham's solution.

Nitrate peptone solution.

Plain broth fermentation tube (control).

Dextrose broth fermentation tube.

Lactose broth fermentation tube.

Saccharose broth fermentation tube.

6. In preparing *agar plate from bacterial cultures*, proceed as follows: Inoculate a tube of nutrient broth *lightly*, using

the *straight* needle. Then, still using the straight needle, from the freshly prepared broth culture, inoculate *lightly* one tube of melted agar (at 40° to 50° C.) and pour into a sterile Petri dish. If the organism shows only a slight growth on the stock culture, transfer directly to melted agar.

7. Moisten a strip of lead acetate paper and insert with cotton plug in tube of Dunham's solution. Blackening of this paper shows the formation of H_2S .

Between what substances does a chemical reaction take place? What are the resulting products?

8. Draw and describe twenty-four-hour cultures of the first four bacteria in all media. If at any time presence of growth is doubtful, compare with a tube of sterile medium. In the absence of growth, reinoculate.

9. Record *macroscopical* changes *only*, in litmus milk; and in fermentation tubes note only, the place of growth, presence and percentage of gas; also the formation of H_2S in Dunham's solution.

10. Make a *permanent stained preparation of each organism* (following directions under Exercise 28). *Young* (twenty-four to forty-eight hour) *cultures must be used*. Use either aqueous-alcoholic fuchsin or aqueous-alcoholic methylen blue.

11. Make a *flagella stain* of the largest motile organism among your cultures.

It is *absolutely necessary* that a *young* (eighteen to twenty-four hour, *not older*) *culture be used* for this purpose. Follow the directions under Exercise 31.

12. Make further drawings and descriptions from day to day *if any change in the growth from that of the preceding day is observed*. Three drawings of a culture will be sufficient. *Endeavor to illustrate typical growth by careful drawings*.

13. State whether the agar plate colony described is a surface or a subsurface colony. How do these two types of colonies differ? Why?

14. Note the presence of condensation water, whether a small or large amount is present. How does this affect colony development?

15. *Draw and measure a typical surface and subsurface colony produced by each organism.*

The form and size often vary with the physical condition under which the colony grows or with physiological conditions, i.e., the proximity of colonies producing poisonous metabolic products.

16. Examine cultures three to six days old in hanging drop for presence of spores. Spores may be seen *free* or enclosed in the bacterial cells. They are easily distinguished by their refractivity. Ordinary anilin dyes will not stain them.

17. Make a *contrast spore stain* of a spore-forming organism. (For method see Exercise 29.)

Draw and describe *only the mature cultures* of the last six organisms (five to eight days old).

18. Make the indol, nitrate and ammonia tests also on the *mature* cultures.

19. In fermentation tube cultures note and record the oxygen requirements of each organism; total per cent of gas; ratio of CO_2 : H_2 and other gases.

20. Test each organism after seven days for indol, nitrate and ammonia production. The culture in Dunham's peptone solution is tested for indol (for method see Exercise 44).

Divide the nitrate peptone solution culture into two parts; test one for nitrates, the other for ammonia (for method see Exercise 45).

21. Prepare permanent stained preparations of one Gram-positive and one Gram-negative organism.

22. Making use of morphological and cultural characteristics ascertained microscopically and by the various cultural tests, identify each organism, using Chester's Manual of Determinative Bacteriology for tracing out

the genus and species. Other valuable reference texts are:

CONN, ESTEN and STOCKING: Classification of Dairy Bacteria.

NOVY: Laboratory Manual of Bacteriology.

JORDAN: General Bacteriology.

EXERCISE 44. EHRLICH'S METHOD OF TESTING INDOL PRODUCTION

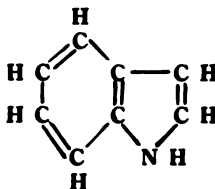
The purpose of the exercise is to test the power of an organism to produce indol from peptone.

Cultures for comparison should be of the same age and grown in the same kind of medium. Some peptones contain a trace of indol and, to avoid all possibility of mistake when testing for indol, a control tube of sterile medium should be used at the same time. This reaction is characteristic for indol or for methyl indol (skatol).

There are other tests for indol, but this one is by far the most delicate. The Salkowski-Kitasato test (conc. H_2SO_4 and NaNO_2) will detect indol in a dilution of only 1 : 100,000, while Ehrlich's test will give a reaction in a dilution ten times greater, or 1 : 1,000,000.

Indol is one of the most important of protein decomposition products. It is noted for its intense fecal odor. However, in highly dilute solutions it has the odor of orange-blossoms, hence is used extensively in perfumery. The jessamine blossom contains indol and has its odor.

Indol has the following graphic formula:



According to Emil Fischer, the reaction of Ehrlich's test, produces, by means of the oxidizing action of the potas-

sium persulphate, a condensation of two molecules of indol with the aldehyde group of the para-dimethyl-amido-benzaldehyde, water splitting off.

Apparatus. Solutions I and II for Ehrlich's test for indol;* two clean 5 c.c. pipettes.

Culture. Dunham's peptone solution or broth culture of the organism to be tested.

Method. 1. To about 10 c.c. of the liquid culture add 5 c.c. of solution I, then 5 c.c. of solution II.

2. Shake the mixture. The reaction may be accelerated by heating. The presence of indol is indicated in a few minutes by a red color which increases in intensity with time. For standard comparisons, five minutes is taken as the maximum time limit.

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- LÖHNIS: Laboratory Methods in Agricultural Bacteriology (1913), p. 42.
- LEWIS, F. C.: On the detection and estimation of bacterial indol and observations on intercurrent phenomena. Jour. Path. and Bact., Vol. 19 (1915), pp. 429-443.

EXERCISE 45. TESTS FOR THE REDUCTION OF NITRATES

The purpose of the exercise is to test the power of an organism to reduce nitrates.

Apparatus. Sulphanilic acid, nitrite test solution I; α -naphthylamin, nitrite test solution II; Nessler's solution; phenolsulphonic acid.

Cultures. Seven-day old nitrate peptone solution cultures grown at 20° to 25° C., or four-day old nitrate peptone solution cultures (pathogenic) grown at 37° C.

Method. (A) *For nitrites:* 1. Add 0.1 c.c. each of solutions I and II to each culture to be tested.

* See Appendix.

2. Repeat with uninoculated control.

3. The development of a red color in ten minutes indicates the presence of nitrites, the intensity of the color depending upon the amount of nitrites present.

(B) *For ammonia.* 1. Add 0.2 c.c. of Nessler's solution to each culture to be tested.

2. Repeat with uninoculated control.

The presence of ammonia is shown by a yellow color or precipitate.

(C) *For nitrates unchanged or free nitrogen liberated.*

1. When either or both of the preceding tests are positive, no further determination need be made, but if negative, then one of two conditions may prevail: (a) Either the nitrates have not been changed, or (b) they may have been reduced to free nitrogen. To ascertain which is true, it will be necessary to determine the presence or absence of nitrates.

2. Test as follows: (a) Evaporate 10 c.c. of each culture and the controls *almost* to dryness in an evaporating dish and add to the residue 1 c.c. of phenolsulphonic acid.

(b) Dilute with 10 c.c. distilled water, then add sufficient ammonium hydroxide, diluted 1 : 1 with distilled water, or concentrated potassium hydroxide solution, to make alkaline.

(c) Transfer the liquid to a 50 c.c. Nessler tube or graduated cylinder and make up the volume to 50 c.c. with distilled water.

A yellow color shows the presence of nitrates.

IN BACTERIOLOGISTS

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(1) For decimal system of group numbers see Table 1. This will be found useful as a quick method of showing close relationships inside the genus, but is not a sufficient characterisation of any organism.

(2) The morphological characters shall be determined and described from growths obtained upon at least one solid medium (nutrient agar) and in at least one liquid medium (nutrient broth). Growth at 37° C. shall be in general not older than 24 to 48 hours, and growths at 20° C., not older than 48 to 72 hours. To secure uniformity in cultures, in all cases preliminary cultivation shall be practiced as described in the revised Report of the Committee on Standard Methods of the Laboratory Section of the American Public Health Association, 1905.

(3) The observation of cultural and bio-chemical features shall cover a period of at least 15 days and frequently longer, and shall be made according to the revised Standard Methods above referred to. All media shall be made according to the same Standard Methods.

(4) Gelatin stab cultures shall be held for 6 weeks to determine liquefaction.

(5) Ammonia and indol tests shall be made at end of 10th day, nitrite tests at end of 5th day.

(6) Titrate with N/20 NaOH, using phenolphthalein as an indicator: make titrations at same times from blank. The difference gives the amount of acid produced.

The titration should be done after boiling to drive off any CO₂ present in the culture.

(7) Generic nomenclature shall begin with the year 1872 (Cohn's first important paper).

Species nomenclature shall begin with the year 1880 (Koch's discovery of the poured plate method for the separation of organisms).

(8) Chromogenesis shall be recorded in standard color terms.

TABLE I.

A NUMERICAL SYSTEM OF RECORDING THE SALIENT CHARACTERS OF AN ORGANISM. (GROUP NUMBER.)

n B.

prins

100.

Endospores produced.

200

Endospores not produced.

LUMINOUS	DISTRIBUTION.	
		Animal pathogen, epizoon
		Plant pathogen, epiphyte
		Soil
		Milk
		Fresh water
		Salt water
		Sewage
		Iron bacterium
		Sulphur bacterium

edna: prompt, gradual, not
...months.

.....
.....

3
is, birds, mice, rats, guinea
pigs, cattle, horses, monkeys,

Group No. (1)

erate, strong, absent, masked

g

1

1. ammonia.....

.. free nitrogen.....

.. strong.

m, slight.

um, slight.

1 bouillon, stated in terms

H moderate, long.

2. exposure in nutrient broth

(of organism)..... C.

with..... C.; or

C., 25° C., 30° C., 37° C.,

H with..... C.

with..... C.

3. t to drying.

2 and crushed ice or liquid

ily sown agar plates; one-

utes), sensitive, not sensitive.

4.....

5.....

6. se, invertase, pectase, cytolase,

7. e, lipase, catalase, glucase,

8.....

II.

1.

d.

Minutes

Temperature

Killing quantity

Amt. required to

restrain growth

2.

Minutes

Temperature

Killing quantity

Amt. required to

restrain growth

3.

Minutes

Temperature

Killing quantity

Amt. required to

restrain growth

BRIEF CHARACTERIZATION

Mark + or O, and when two terms occur on a line erase the one which does not apply unless both apply.

MORPHOLOGY. (2)

Diameter over 1μ

Chains, filaments

Endospores

Capsules

Zoogloea, Pseudozoogloea

Motile

Involution forms

Gram's Stain

Cloudy, turbid

Ring

Pellicle

Sediment

Shining

Dull

Wrinkled

Chromogenic

Round

Protens-like

Rhizoid

Filamentous

Curled

Surface-growth

Needle-growth

Moderate, absent

Abundant

Discolored

Starch destroyed

Grows at 37° C.

Grows in Cohn's Sol.

Grows in Uschinsky's Sol.

Gelatin (4)

Blood-serum

Casein

Agar, mannan

Acid curd

Rennet curd

Casein peptonized

Indol (5)

Hydrogen sulphide

Ammonia (5)

Nitrates reduced (5)

Fluorescent




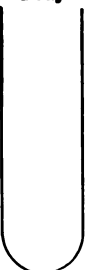

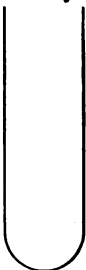
CULTURAL FEATURES. (3)

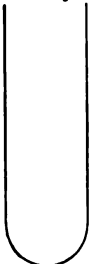
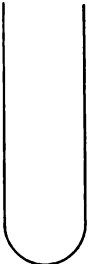

BIOCHEMICAL FEATURES.




TESTS FOR THE REDUCTION OF NITRATES 135

BACTERIA

Name of student		Desk No.
Name of organism	Isolated from	
Method of isolation		
Occurrence		
Importance		
Shape of organism	Arrangement	Size
Motility	Flagella	
Method of reproduction	Involution forms	
Spore	Stages of germination	
Aqueous-alcoholic stain	Gram's stain	Acid-fast stain

	1 daydaysdays
Agar streak			
Titre.....			
Incubated at			
..... ° C.			
	1 daydaysdays
Gelatin stab			
Titre.....			
Incubated at			
..... ° C.			

	1 daydaysdays
Broth culture			
Titre			
Incubated at			
..... ° C.			

	1 daydaysdays
Potato culture			
Titre			
Incubated at			
..... ° C.			

Age of agar colonydaysdaysdays
Size of colony			
Surface elevation			
Agar colony Titre			
Incubated at			
..... ° C.			

TESTS FOR THE REDUCTION OF NITRATES 137

Age of gelatin colonydaysdaysdays
Size of colony			
Surface elevation			
Gelatin colony Titre..... Incubated at ° C.			

Litmus milk	Acid	
	Gas	
	Acid curd	
	Rennet curd	
	Reduction	
	Alkali	
	Peptonization	

Fermentations

% Of gas in	Control	Dextrose	Lactose	Saccharose	Glycerin
24 hours					
48 hours					
3 days					
7 days					
Total gas production					
Ratio of CO ₂ :H ₂ and other gases					
Acid					
Growth in closed arm					

Chromogenesis on	Nutrient broth	
	Nutrient gelatin	
	Nutrient agar	
	Potato	
Production of	NH ₃ from peptone	
	H ₂ S from peptone	
	Indol from peptone	
	Nitrites from peptone	
Reduction of nitrates to	NH ₃	
	Nitrites	

Remarks:

EXERCISE 46. TO DEMONSTRATE THE EFFICIENCY OF INTERMITTENT HEATING AS A METHOD OF STERILIZING MEDIA. ALSO TO COMPARE THE EFFICIENCY OF CONTINUOUS AND INTERMITTENT HEATING

Apparatus. 400 c.c. fresh skim milk; forty sterile test tubes; 2% azolitmin solution.

Method. 1. Prepare litmus milk according to directions on p. 25.

2. Fill the tubes, using approximately 8 c.c. per tube.

3. Set five away without heating.

Heat five for fifteen minutes on the first day.

Heat five for one hour on the first day.

Heat five for fifteen minutes on two successive days.

Heat five for fifteen minutes on three successive days.

Heat five for fifteen minutes on four successive days.

Heat ten for fifteen minutes on five successive days.

4. Keep all tubes at room temperature. Examine every two or three days and describe the macroscopical changes of each set, as described under the discussion on litmus milk (pp. 23-25.)

Why do not all the tubes of a set change alike? Why do not all sets present the same appearance?

Save all tubes that do not show macroscopical changes. These are probably sterile.

5. Tabulate your results after ten days to two weeks, recording the number and per cent of each lot that shows macroscopical changes.

6. Is milk difficult to sterilize? Why? What other media present the same problem of sterilization as milk? Why?

Would any other method for the sterilization of milk be preferable to the ones you used? Give reasons for your answer.

7. State your results in detail and point out any conclusions that may be drawn and any practical applications that may be made.

REFERENCES

- MARSHALL: Microbiology, pp. 153-161, 306-313, 363-365.
CONN, H. W.: Bacteria, Yeasts and Molds, pp. 191-193.
BESSON, A.: Practical Bacteriology, Microbiology and Serum Therapy, pp. 35-36.

**EXERCISE 47. TO COMPARE MORPHOLOGICALLY
PROTOZOA WITH BACTERIA**

Apparatus. Deep culture dish; concave slide; clean cover-glasses; cover-glass forceps; platinum loop; tube of sterile broth; tube of sterile Chinese ink.

Cultures. Rich soil or slimy leaves from a pond.

Method. 1. Place the soil or leaves in the deep culture dish.

2. Fill the dish two-thirds full with tap water and add the contents of a tube of broth.

3. Keep the dish at room temperature for twenty-four to forty-eight hours.

4. At the end of the incubation period, make a hanging drop from the supernatant liquid. Before inverting the drop on the slide, add to it that amount of Chinese ink that adheres to the end of a platinum needle.

By the use of this ink, organisms are brought out by contrast, showing white on a dark field. The organisms are not killed or injured by the ink.

5. Observe and measure any protozoa, using the lowest power objective with the step micrometer. Record the size in micra.

6. Roughly sketch the different species observed, giving comparative measurements.

7. Using the highest power dry objective, observe bacteria, noting morphology and size.

8. Draw lines to represent the ratio between the size of the predominant types of each.

9. Are the protozoa present visible to the naked eye?

How many of the largest protozoa present, placed end to end, would make an inch?

10. How do the protozoa and bacteria in the drop compare in numbers? Do these organisms have any relation to each other? If so, explain.

11. Of what importance are protozoa? Name several well-known protozoa.

12. State your results in detail and point out any conclusions that may be drawn and any practical applications that may be made.

REFERENCE

MARSHALL: Microbiology, pp. 10-11, 68-80, 82-84.

EXERCISE 48. TO STUDY THE NATURAL DECOMPOSITION OF MILK

Apparatus. 500 c.c. sterile Erlenmeyer flask; two 5 c.c. sterile pipettes; ten 1 c.c. sterile pipettes; four 10 c.c. sterile pipettes; six 200 c.c. sterile Erlenmeyer flasks; fifteen sterile Petri dishes; physiological salt solution.

Cultures. Fresh skim milk.

Method. 1. Prepare "dilution flasks" as given in Exercise 13, p. 52, making two 90 c.c. and four 99 c.c. flasks. Sterilize by heating one hour in flowing steam or five minutes in the autoclav at 120° C. (15 lbs. pressure). *Dilution flasks and all glassware must be sterile before the experiment proper can be started.*

2. Place 200 c.c. of the fresh skim milk in the sterile 500 c.c. flask and use this sample for the entire experiment.

3. Plate the milk immediately on nutrient agar, using dilutions according to the age of the milk, as follows. (See Exercise 13, p. 52, for method of using dilution flasks.)

Age.	Dilutions.
Fresh milk	1 : 1,000, 1 : 10,000 and 1 : 100,000
One day old	1 : 10,000, 1 : 100,000 and 1 : 1 M *
Four days old	1 : 10 M, 1 : 100 M and 1 : 1,000 M
Eight days old	1 : 10 M, 1 : 100 M and 1 : 1,000 M
Ten days old	1 : 1 M, 1 : 10 M and 1 : 100 M

Keep the plates at room temperature.

Sterile pipettes are to be used *always* in making dilutions, plating and titrating.

After the milk curdles it is advised to make the first dilution 1 : 10 to give a more uniform sample, from which further dilutions are made. Use a 10 c.c. pipette having a large opening in the delivery end to prevent clogging.

4. Titrate the milk sample every day. After the milk curdles, shake well before titrating and choose a 5 c.c. pipette having a large aperture for delivery for obtaining the sample for titration.

5. Record the reaction in degrees of Fuller's scale.

After using pipettes, dilution flasks, etc., clean, refill and sterilize them at once for future use.

6. Note the *macroscopical changes* in the milk sample (due to microbial growth), e.g., kind and consistency of curd, extrusion of whey, gas formation, peptonization; also note odor from time to time.

7. Note the *macroscopical evidences of microbial growth* such as molds, etc., and the time of appearance. Identify the group to which these organisms belong, giving genus and species if possible.

8. Determine the changes in the numbers of micro-organisms by counting the colonies of the different sets of plates after they have developed seven days at room temperature (see p. 56, Exercise 14, for method).

9. Estimate the number of colonies of each type (see Exercise 14, p. 56).

* M = Million.

10. Record your results, noting the *date* on which the plates were made, the *age* of the milk, the *dilution*, *number* of colonies *on the plate* and the *average number* of organisms *per cubic centimeter*.

11. Examine in hanging drop and note the morphology of the microorganisms producing the most predominant types of colonies on each set of plates. Indicate after the drawing, the comparative numbers on each set of plates by the signs -, +-, +, ++, etc., to indicate absence, presence of few, or many of the type.

12. Note whether molds or yeasts are present on any set of plates. Should either be found on fresh milk plates? Why? What types of microorganisms would you expect on fresh milk plates?

13. Prepare your data according to the following diagram:

Date	Age	Acidity	Dilution	Count per cc.	Organisms	
					Types	Relative Nos.
Feb. 2	Fresh (26 hrs. old)	+15°	1-1,000	278,900	Acid	+
			1-10,000	325,500	Yellow	+ -
			1-100,000	300,000		

Average count per cc..... 301,470

14. Plot the curve showing the change in acidity and one illustrating the count per cubic centimeter on the same paper, using different colored inks or different types of lines. Use days for abscissæ, acidity and count for ordinates. Start at the same origin.

15. Is there any relation between the change in acidity and the change in flora?

Should the acidity and count curves run parallel? If they do not, give a reason why.

How could the bacterial count be made to increase after it goes down to a constant number?

16. What biochemical changes have occurred in the decomposition?

17. Compare the flora of fresh milk, 70° acid milk and ten-day milk, both microscopically and from the plates. Explain.

18. State your results in detail and give any conclusions to be drawn and any practical applications that may be made.

REFERENCES

CONN: Practical Dairy Bacteriology, pp. 21-57, 81-85.

MARSHALL: Microbiology, pp. 298-299, 306-313, 321-326.

EXERCISE 49. TO ISOLATE SPORE-FORMING BACTERIA AND TO STUDY SPORE FORMATION

Apparatus. Two tubes of sterile broth; small piece of hay; three sterile Petri dishes; clean test tube; three tubes sterile agar for plates; three sterile agar slants; carbol-fuchsin; acetic acid alcohol; aqueous-alcoholic methylen blue; platinum needle and loop; ordinary forceps.

Culture. Hay.

Method. 1. Place a piece of hay in the clean test tube, plug the tube, and sterilize in the hot-air oven.

2. Using sterile forceps, place the sterile hay "aseptically" in one tube of broth and an unsterilized piece in the other.

3. Incubate both at room temperature for forty-eight hours. Do both tubes show growth?

4. Heat in a water-bath at 80° C. for ten minutes the tube which shows marked growth. What does this accomplish?

5. Make three loop-dilution plates from the heated broth tube.

6. Place the plates at room temperature and examine them daily for colony development.

REMOVING MICROORGANISMS FROM LIQUIDS 145

7. Make pure cultures on agar slants from three different well-isolated colonies of the predominant types and incubate at room temperature.

8. Examine these in thirty-six to forty-eight hours in a hanging drop for morphology and spore formation.

9. Make a spore stain as soon as spores are found. Where are the spores located in the bacterial cell?

10. Have you studied any pure culture of bacteria which is similar to the types you have isolated? What organism is commonly found in hay? In what form does it exist on the hay? What do you know of the habitat of this organism and related forms? Of the pathogenicity?

11. State the results obtained in detail; draw the conclusions which follow and point out any possible practical applications.

REFERENCES

MARSHALL: Microbiology, pp. 5, 45, 154, 189, 242.

JORDAN: General Bacteriology, 4th Ed., pp. 235-236.

EYRE: Bacteriological Technic, 2d Ed., p. 140.

EXERCISE 50. TO DEMONSTRATE THE EFFICIENCY OF FILTRATION AS A MEANS OF REMOVING MICROORGANISMS FROM LIQUIDS

Apparatus. Six small funnels; two small filter papers; two small pieces of absorbent cotton; two small pieces of clean hospital gauze; eight tubes sterile agar; eight sterile Petri dishes; ten sterile 1 c.c. pipettes; sterile 10 c.c. pipette; three dilution flasks; six sterile test tubes; tube of sterile broth.

Culture. *B. coli*.

Method. 1. Inoculate broth with *B. coli* and incubate for twenty-four hours at 21° C.

2. Sterilize filter paper in each of the two small funnels, a small piece of absorbent cotton in each of two more; fold two pieces of clean gauze several thicknesses and

sterilize in the remaining two funnels. Wrap all in paper and sterilize in the hot-air oven.

3. Shake the broth culture of *B. coli* and plate, using dilutions 1 : 1,000 and 1 : 10,000.

4. Filter each dilution (1 : 1,000 and 1 : 10,000) through each of the different substances, catching the filtrate in sterile test tubes.

5. Plate 1 c.c. from each filtrate immediately and incubate the plates at 21° C.

6. At the end of five days, count the plates.

7. Which method of filtration is most efficient? Why? What factors could greatly influence the numbers of microorganisms developing on the plates after filtration?

8. What methods are most efficient in removing microorganisms from liquids? Why?

9. Suggest some natural methods of filtering microorganisms.

10. Give in detail the results obtained, state any conclusions that may be drawn and point out any practical applications.

REFERENCES

EYRE: Bacteriological Technic, 2d Ed., pp. 42-48.

MARSHALL: Microbiology, pp. 64-67.

EXERCISE 51. TO DEMONSTRATE PRESENCE OF MICROORGANISMS IN AIR, ON DESK, FLOOR, ETC.

Apparatus. Seven sterile Petri dishes; seven tubes of sterile agar.

I. *Air*. **Method.** 1. Pour seven plates with uninoculated sterile agar and set on a level surface until solid.

2. Expose one plate for one minute to (a) laboratory air; (b) air of campus; (c) air of your room while sweeping or dusting.

II. *Floor*. 1. Bend the straight platinum needle till it forms a right angle.

2. Sterilize it in the flame.
3. Moisten the needle with sterile water.
4. Rub it along the floor, and then,
5. Draw it lightly across the surface of the agar in the fourth Petri dish.

III. *Desk.* 1. Sterilize the needle and repeat operation (II, 5) obtaining the inoculum from the surface of a desk which has *not* just previously been washed with 1 : 1,000 mercuric chloride.

2. Then wash the surface of the desk well with this solution and when the desk top is dry, repeat the operation, using the sixth plate. Leave the seventh plate uninoculated for control.

3. Mark all plates with the date on which they were exposed or inoculated and place them at a constant temperature.

4. Watch any developments from day to day. What organisms predominate on the plates? Why?

5. Examine different colonies in a hanging drop. What types of bacteria are found?

6. Upon what does the species and number of microorganisms depend? What becomes of them when air currents are present? When the floor is swept in the ordinary way? Mopped? When the desk is washed with water? With mercuric chloride?

7. Are these types deleterious to health? Why should and how may they be avoided in the laboratory? Outside of the laboratory?

8. State your results for I, II and III in detail, draw any conclusion possible and point out any practical operations.

REFERENCES

- MARSHALL: Microbiology, pp. 185-191.
BESSON: Practical Bacteriology, Microbiology and Serum Therapy, pp. 862-863.
CONN: Bacteria, Yeasts and Molds, pp. 114-123.
CONN: Practical Dairy Bacteriology, pp. 65-67.
TYNDALL: Floating Matter of the Air.

EXERCISE 52. QUALITATIVE STUDY OF THE MICRO-FLORA OF THE SKIN AND HAIR IN HEALTH AND IN DISEASE

Apparatus. Ordinary forceps; two sterile, small white enamel basins (*steam-sterilized*); three sterile 1 c.c. pipettes; six sterile Petri dishes; six tubes of sterile agar; one liter flask containing about 700 c.c. of sterile water or salt solution; sterile cloth ($\frac{1}{2}$ yd. hospital gauze wrapped in paper and sterilized at 180° C.); soap; clean slides and cover-glasses.

Method. I. *Skin.* (a) *Normal.* 1. Place about half the sterile water in a sterile basin.

2. Wash the hands thoroughly in the sterile water.

3. Plate 1 c.c. of this water immediately in ordinary agar.

4. Then wash the hands well with soap and tap water, rinse with tap water till free of soap and dry the hands on the sterile cloth.

5. Place the remaining sterile water in the second sterile basin and wash the hands again.

6. Plate 1 c.c. of this water.

7. Incubate both plates (inverted) at 37° C.

8. How long before starting this experiment did you wash your hands? How might this influence your results?

9. What types of microorganisms would you expect to find on the skin? Why?

(b) *Diseased.* 1. With a sterile needle obtain a small amount of purulent material from a pustule, boil, or abscess, etc.

2. Make three loop-dilution plates in agar. Incubate at 37° C.

3. Examine some of this material microscopically by preparing a stained smear.

4. Draw and describe the latter. Do you find the same organisms on the plates as on the slide?

5. *Isolate the most predominant organisms on the plates and identify them.*

6. What is the source of all these microorganisms? What becomes of them when we wash our hands and wipe them in the ordinary way? Are they detrimental to health?

7. What is pus? Of what does it consist? What care should be taken with discharges from suppurating sores?

II. *Hair.* (a) *Normal.* 1. Using flame-sterilized forceps (ordinary type), obtain several hairs and place them in a sterile Petri dish.

2. Using a sterile pipette, add 1 c.c. of sterile water or salt solution to the Petri dish and stir the hairs about in it with the pipette or sterile loop in order to dislodge the organisms adhering to them.

3. Pour into the plate a tube of melted agar (at 40° to 45° C.), and when hard, incubate at 37° C.

4. After twenty-four to forty-eight hours, examine predominating colonies in a hanging drop.

(b) *Diseased.* 1. With sterile forceps obtain a few hairs from the growing edge of the infected portion of the skin affected with ringworm or barber's itch. These hairs will come out easily in comparison with healthy hairs.

2. Mount and examine for the fungus, *Trichophyton tonsurans*. (See illustration on p. 578 in Marshall's Microbiology.) Draw.

3. Continuous application of a glycerinated solution of 1 : 500 HgCl₂ (glycerin 1 part, HgCl₂ 1 : 500, 9 parts) will kill this fungus.

State in detail your results for I and II, draw any conclusion permissible and point out any practical application.

REFERENCES

- MARSHALL: Microbiology, pp. 522-524, 545, 578, 591-593.
 BESSON: Practical Bacteriology, Microbiology and Serum Therapy, pp. 679-688.

EXERCISE 53. QUALITATIVE STUDY OF THE MICRO-FLORA OF THE MUCOUS MEMBRANE (MOUTH AND THROAT OR NOSE)

Apparatus. Absorbent cotton, small piece; wire rod about 15 cms. long; clean slides and cover-glasses; Petri dish, sterile; tube of sterile agar; tube of sterile broth; aqueous-alcoholic fuchsin.

Method. I. For Teeth. 1. Place a small drop of distilled water on a clean cover-glass or slide.

2. Introduce some material obtained by scraping along the base of and between the teeth with a sterile platinum needle.

3. Allow to dry, fix and stain with aqueous-alcoholic fuchsin.

4. Examine microscopically with the oil immersion lens.

5. Draw all forms seen. Would all of these forms grow on an agar plate? Give reasons for your answer.

II. For Throat or Nose. 1. Prepare a swab by winding a small piece of absorbent cotton snugly about one end of the wire rod.

2. Place in a test tube, swab end down, and prepare for sterilization as with pipettes.

3. Dry-sterilize.

4. Pour an agar plate and allow it to harden.

5. Moisten the sterile swab in sterile broth, using aseptic precautions, and then swab the throat or nose.

6. Lightly brush the inoculated swab over the surface of the agar plate and place the plate inverted, at 37°, to develop.

7. Using the same swab, make a smear on a clean glass slide, dry, fix, stain and examine as with the preparation from the teeth.

8. Return the swab to the tube of broth, incubate at 37° for twenty-four hours and examine the growth in a hanging drop.

9. Draw and describe the predominating organisms.

10. Find a streptococcus, if possible, on the agar plate from the swab.

11. Make a stained slide and have the instructor inspect the same, when you think that you have been successful.

12. How does the microflora of the mucous membrane differ from that of the outer skin?

CAUTION. Aseptic precaution must be taken in all instances, as some of the microorganisms may be pathogenic!

State in detail your results from I and II, draw any conclusions possible and point out any practical applications.

REFERENCES

- MARSHALL: Microbiology, pp. 522-524, 528, 546, 591-599, 609-613.
BESSON: Practical Bacteriology, Microbiology and Serum Therapy,
pp. 81, 191, 197, 270, 592-610, 617-626.

PART II

PHYSIOLOGY OF MICROORGANISMS

EXERCISE 1. TO DEMONSTRATE THE SMALL AMOUNT OF FOOD NEEDED BY BACTERIA

Apparatus. Distilled water; eleven sterile Petri dishes; sterile 1 c.c. pipette; sterile 10 c.c. pipettes; eight sterile 200 c.c. flasks; eleven tubes of sterile agar (ordinary).

Cultures. *B. coli*.

Method. 1. Place 150 c.c. of distilled water in each of two sterile flasks.

2. Sterilize one flask (flask *B*) in the autoclav for ten minutes at 15 lbs. pressure (120° C.).

3. Plate 1 c.c. from the remaining flask (flask *A*), immediately on agar.

4. As soon as flask *B* is cold, plate 1 c.c.

5. Then inoculate the water in flask *B* with *B. coli*, using the *straight needle and transferring a very small amount*, and plate 1 c.c.

6. Place flasks *A* and *B* and the plates made from the flasks at room temperature.

7. Prepare four 90 c.c. and two 99 c.c. dilution flasks and sterilize.

8. At the end of five days, plate from flasks *A* and *B*, using 1 c.c. direct and dilutions 1 : 10, 1 : 100 and 1 : 1000. Incubate the plates at room temperature.

9. Count each set of plates at the end of five days' incubation.

10. Compute the weight of the bacteria in the flask of distilled water at its highest count.

What is the smallest amount that may be weighed on the ordinary analytical balances? Conclusions?

11. Plot curves to show whether bacteria are decreasing or increasing. Offer a logical explanation for the direction the curve takes in each instance.

12. Note the conditions under which distilled water is obtained and dispensed in the laboratory. Why is the distilled water not sterile?

13. By what process of distillation may distilled water be obtained free from microorganisms? What several factors outside of errors in technic may have influenced your results?

14. What would be the comparative influence of a large and a small inoculation upon the number of *B. coli* surviving the 5 days sojourn in the distilled water?

15. State your results in detail, draw any possible conclusions and point out any practical applications.

REFERENCES

MARSHALL, C. E.: Microbiology, pp. 88-89.

FISCHER, ALFRED: Structure and Functions of Bacteria, pp. 52-54.

PRESCOTT and WINSLOW: Elements of Water Bacteriology, 3d Ed. pp. 151-153.

SOME PHYSIOLOGICAL CLASSIFICATIONS OF BACTERIA

Bacteria are often classified, in general terms, **according to their functions**, into:

Saprogenic, or putrefactive bacteria;

Zymogenic, or fermentative bacteria;

Pathogenic, or disease-producing bacteria.

According to their food requirements, into:

Prototrophic, requiring no organic food (e.g., nitrifying bacteria);

Metatrophic, requiring organic food (e.g., zymogenic bacteria, saprophytes and facultative parasites);

Paratrophic, requiring living food (e.g., obligate parasites); (A. Fischer).

According to special food preferred, into:

Acidophile: acid loving;

Halophile: salt loving;

Saccharophile: sugar loving;

Saprophile: loving dead organic matter;

Coprophile: loving barnyard manure.

According to their oxygen requirements, into:

Aerobic: requiring atmospheric oxygen for growth;

Anaerobic: requiring the absence of atmospheric oxygen;

Partial anaerobic: requiring an intermediate oxygen tolerance.

According to the necessity of one kind of food or environment, into:

Obligate: indicating absolute requirements, e.g., obligate parasite, obligate anaerobe;

Facultative: indicating a variability in requirements; the word following indicates the condition under which the organism may live but does not prefer for growth, e.g., *B. coli* is a facultative anaerobe.

According to their metabolic products, into:

Chromogenic, or pigment-producing bacteria;

Photogenic, or light-producing bacteria;

Aerogenic, or gas-producing bacteria;

Thermogenic, or heat-producing bacteria.

Chromogenic bacteria are classified in accordance with the nature and location of the coloring matter which they elaborate, as

Chromophorus bacteria, the pigment being stored in the cell protoplasm of the organism analogous to the chlorophyll of higher plants, e.g., green bacteria and red sulphur bacteria, purple bacteria.

Chromoparous bacteria, true pigment formers. The pig-

ment is set free as a useless excretion, may be excreted as a colored body or as a colorless substance which becomes oxidized upon exposure to the air. Individual cells are colorless and may cease to form pigment, e.g., *B. prodigiosus*, *B. ruber*, *B. indicus*.

Parachrome bacteria. The pigment is an excretory product but is retained within the cell, e.g., *B. violaceus*. (Bejerinck.)

According to their temperature relations, into:

Pecilothermic (*poikilothermic*) bacteria: adaptability to temperature of environment;

Stenothermic bacteria: a very narrow temperature range (strict parasites);

Eurythermic bacteria: a very wide temperature range (metatrophic bacteria), often 30° between maximum and minimum temperatures.

According to their optimum temperature, into:

<i>Cryophilic</i> (<i>psychrophilic</i> , term used chiefly for water organisms) bacteria.....	Min. 0° C.	Opt. 15° C.	Max. 30° C.
<i>Mesophilic</i> bacteria (includes pathogenic bacteria).....	15° C.	37° C.	45° C.
<i>Thermophilic</i> bacteria.....	45° C.	55° C.	70° C.

ANAEROBIC CULTURE METHODS

The cultivation of strict anaerobes is accompanied by certain technical difficulties arising from the necessity of removing all traces of oxygen from the medium and from the atmosphere to which this medium is exposed. It is, therefore, necessary to employ special apparatus or special methods for their cultivation.

The recent investigations of Tarrozzi, which have been confirmed by others, seem to show that oxygen does not exert any direct harmful effect on anaerobic organisms, but that the presence of free oxygen prevents the medium furnishing

the nutritive substances necessary for anaerobic life. Anaerobic organisms can, in fact, as Tarrozzi has shown, be grown in the presence of the oxygen of the atmosphere by simply adding pieces of animal tissue or some reducing agent to the culture medium.

Several principles are employed as a basis for the different methods of anaerobic cultivations, as follows:

I. *Exclusion of air* from the cultivation.

II. *Exhaustion of air* from:

1. The medium by boiling. This should always immediately precede the inoculation of the medium for anaerobic cultivations.

2. The vessel containing the medium by means of an air pump, i.e., cultivation *in vacuo*.

III. *Absorption of oxygen* from the air in contact with the cultivation, i.e., cultivation in an atmosphere of nitrogen, by means of:

1. Chemical action upon a readily oxidizable substance in a sealed vessel containing the cultures, e.g., sodium hydroxide upon pyrogalllic acid.

2. Burning a filter paper saturated with alcohol in a sealed vessel. (Moore.) If the paper is well saturated no deleterious products of combustion are formed which would inhibit growth.

3. Adding to the medium some easily oxidizable substance as dextrose (2%), sodium formate (0.5%), sodium sulphindigotate (0.1%) or fragments of sterile tissue to absorb all the available oxygen held in solution by the medium.

The chemicals are generally employed in the case of deep stab cultures, the fragments of sterile tissue in broth cultures (Tarrozzi's method). The tissue must be freshly removed from an animal (rabbit, mouse, guinea pig, etc.) and only pieces of liver, spleen, kidney or lymphatic glands may be used with success; blood, milk, or the connective tissues are useless for the purpose. Vegetable tissue (potato,

elder pith, mushrooms, etc.) have been used similarly with success (Wrzosek, Ori and others). Spongy platinum has also been used similarly with satisfactory results.

The vitality of anaerobic organisms is exhausted much more quickly on media prepared on these principles than on media under anaerobic conditions (Jungano and Distaso).

Perhaps if these methods were used in conjunction with anaerobic methods the vitality of the anaerobes would not be impaired.

4. Growing the anaerobe in the presence of a vigorous aerobe by the use of special methods or apparatus.

IV. *Displacement of air* by an indifferent gas such as hydrogen, carbon dioxide, etc.

V. *A combination of two or more of the above methods.*

The following methods are those best adapted for class use and can be utilized in a regular exercise as desired:

I. EXCLUSION OF AIR

Hesse's Method. This method may be used either with a pure culture or for determining the presence of anaerobes in any substance.

Apparatus. Tubes of agar or gelatin for stab cultures; sterilized oil (olive oil, vaselin or paraffin oil); sterile 1 c.c. pipette.

Culture. Pure culture of an anaerobe.

Method. 1. Make a stab culture of the anaerobe, using a tube containing a deep column of the medium, and thrusting the inoculating needle to the bottom of the tube. The stab culture and a test tube shake culture also may be treated as follows:

2. With the sterile pipette place a layer of sterile oil,* 1 to 2 cm. deep, upon the surface of the medium.

3. Incubate at the optimum temperature.

* Sterile melted agar or gelatin may be substituted for the sterile oil.

II. EXHAUSTION OF AIR

A. By Boiling. It is well to expel all the air from a medium to be used for isolating or growing anaerobes by boiling twenty to thirty minutes, and cooling rapidly just previous to inoculating, and placing under anaerobic conditions.

B. Cultivation in Vacuo. This requires special apparatus for obtaining a vacuum and for cultivation in some cases.



FIG. 39a.—Novy Jar for Tube Cultures.

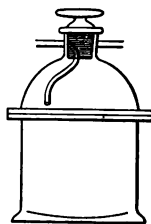


FIG. 39b.—Novy Jar for Plate Cultures.

Apparatus. *Special tubes:*

1. Vacuum tubes (Fig. 129, p. 238, Eyre's Bacteriological Technic).
2. Pasteur, Joubert and Chamberland's tube (Fig. 80, p. 93, Besson's Practical Bacteriology, Microbiology and Serum Therapy).
3. Pasteur's tube (Fig. 81, Besson, *ibid.*).
4. Lacomme's tube (Fig. 82, Besson, *ibid.*).
5. Roux's tube for stroke cultures (Fig. 91, p. 101, Besson, *ibid.*).
6. Roux's tube for potato cultures (Fig. 92, p. 101, Besson, *ibid.*).
7. Esmarch's tube (Fig. 95, p. 103, Besson, *ibid.*).
8. Vignal's tube (Fig. 96, p. 103, Besson, *ibid.*).

Special flasks:

1. Pasteur's flask (Fig. 79, p. 92, Besson, *ibid.*).
2. Flasks with long necks (Fig. 83, p. 94, Besson, *ibid.*).
3. Bottle (Fig. 84, p. 94, Besson, *ibid.*).
4. Kitasato's dish (Fig. 93, p. 10, Besson, *ibid.*).
5. Bombicci's dish (Fig. 94, p. 102, Besson, *ibid.*).
6. Ruffer's or Woodhead's flask (Fig. 33, p. 41, Eyre, *ibid.*).

Special jars in which test tube or plate cultures may be placed and a vacuum produced.

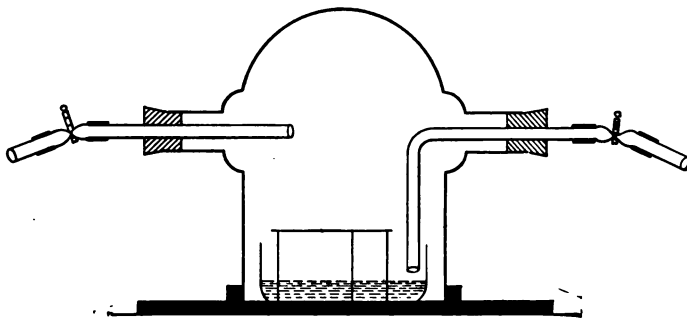


FIG. 40.—Bulloch's Anaerobic Jar.

1. Novy's jar for plates (Fig. 135, p. 245, Eyre, *ibid.*).
2. Novy's jar for tubes (Fig. 136, p. 245, Eyre, *ibid.*).
3. Bullock's anaerobic apparatus (Fig. 137, p. 247, Eyre, *ibid.*).

4. Tretrôp's apparatus (Fig. 97, p. 105, Besson, *ibid.*).

5. Botkin's apparatus (Fig. 134, p. 244, Eyre, *ibid.*).

Apparatus for obtaining a vacuum:

1. Electric pump adaptable to vacuum or pressure.
2. Water vacuum pump.
3. Mercury vacuum pump.

Method. 1. The tube and flask cultivations are prepared by, (a) placing the desired medium in the vessel; (b) inoculating from the desired source; (c) attaching to the vacuum

pump and (d) while the pump is running, sealing the tube or flask in the flame, at the constriction provided for the purpose.

2. The special jars have the advantage that tube and plate cultivations may be prepared in the usual way and then placed in the special jar which is then attached to the vacuum pump; when sufficient vacuum has been produced the stopcock is turned between the jar and the pump.

Isolation of anaerobic organisms may be accomplished with much greater facility by the use of these jars.

In practically every instance these same jars may also be employed in the methods given under the absorption of oxygen.

III. ABSORPTION OF OXYGEN

Different methods illustrating this general principle are much used because of its simplicity and general applicability. Any vessel with a tight cover as a Novy jar, an ordinary chemical desiccator, a Mason fruit jar, etc., may be used as a container for the tube or plate culture.

A. Pyrogallic acid method. 1. Dry pyrogallic acid is placed on top of some absorbent cotton in the bottom of the jar or tube.

2. A solution of sodium hydroxide is poured in, but not directly upon it.

3. The cultures are put in place.

4. The jar or tube is immediately sealed and care is taken to mix the chemicals. The organisms thus grow in the presence of the inert gas nitrogen.

The chemicals are used in the proportion of 1 gm. of pyrogallic acid to 10 c.c. of 10% aqueous solution of potassium or sodium hydroxide for each 100 c.c. of air space.

Apparatus. *Tubes* for use in oxygen absorption method.

1. Simple test-tube method.

2. Giltner's H tube.

3. Buchner's tube (Fig. 130, p. 239, Eyre, *ibid.*).

4. Turro's tube (Fig. 86, p. 95, Eyre, *ibid.*).

By the use of these tubes no sealed jar is necessary.

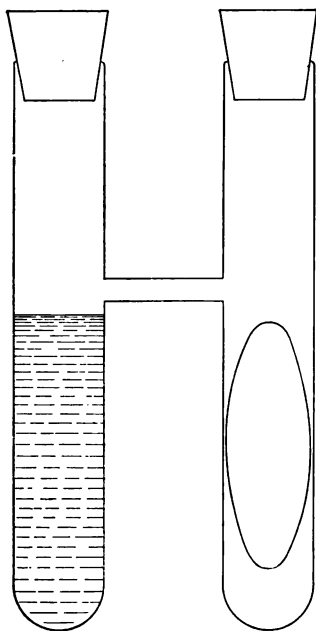


FIG. 41.—Giltner's Tube. (Orig.)

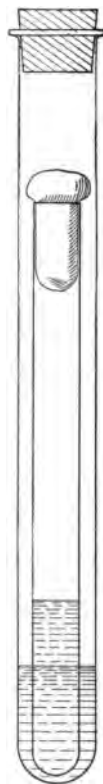


FIG. 42.—Buchner's Tube.

1. The simple test-tube method is advantageous in that it requires no special apparatus. It has disadvantages, however, which will be mentioned later.

Apparatus. Test tube of sterile medium; rubber stopper to fit tube; pyrogallic acid and sodium hydroxide; paraffin.

Method. 1. Inoculate the medium with the material under investigation and replace the plug.

2. Cut off the plug even with the mouth of the tube.

3. Push the plug into the tube, 4 to 5 cm.

4. Place on top of the plug the pyrogalllic acid and *only enough* of the alkaline solution to saturate the plug.

5. Insert the rubber stopper and seal with paraffin if necessary. If the cotton is more than saturated, the strong alkaline solution will run through the plug and kill the organisms in the culture.

This preparation is valuable only for noting the presence of anaerobes in any substance or studying the growth of an anaerobe in pure culture, on account of the difficulties of technic.

2. *Giltner's H Tube.* This is simply two test tubes connected near their mouths by a short piece of glass tubing. By this method the tube cultivation may be placed in one test tube, the chemicals in the other and both tubes stoppered. (Fig. 41, p. 161).

The use of this apparatus presents a distinct advantage over any other tube cultivation method, as the culture is readily discernible at all times and may be handled without the disagreeable features of the other methods.

The H tube lends itself also to the method depending upon the absorption of oxygen by an aerobic organism.

3. *Buchner's tube* consists of a stout glass test tube having dimensions of about 23 cm. in length and 4 cm. in diameter, fitted with a rubber stopper.

a. A test-tube culture of the organism or mixed culture to be tested is prepared.

b. A little cotton, the pyrogalllic acid, and sodium hydroxide solution are placed in the Buchner tube, the culture *immediately* introduced and the rubber stopper *immediately* fitted tightly in the mouth of the large tube. (Fig. 42, p. 161).

4. *In Turro's tube*, the medium is poured through the

small inner tube, sterilized and inoculated. The pyrogallie acid and sodium hydroxide are then placed in the bulb and the stopper immediately replaced.

This method has advantages over Buchner's in that the oxygen is much more rapidly absorbed and the culture is visible during incubation.

Plates. 1. Ordinary deep culture (Petri) dish.

2. McLeod's plate base (used with the bottom of a deep Petri dish). (Muir and Ritchie, 6th Ed., Fig. 23, p. 66.)

The principle of using these two plates is the same throughout and is illustrated in Exercise 2.

Jars. As has been noted before, the jars designed for obtaining vacuum may be utilized in the pyrogallie acid method and in the method making use of burning alcohol to exhaust the oxygen.

B. Liborius-Veillon Method and Roux's Biological Method depend upon the *abstraction of oxygen from the medium by aerobic organisms*. Liborius makes use of the aerobes already present in the mixed culture, while Roux uses a pure culture of an obligate aerobe. Nowak first grew *Bact. abortus* by this method.

Liborius-Veillon Method. 1. Fill long test tubes (22 cm. \times 1.5 cm.) to a depth of 10–15 cm. with glucose agar or gelatin and sterilize (below 120° C.).

2. Place the tubes in a water bath and boil twenty to thirty minutes to liquefy the agar and drive off the air dissolved in the medium; then cool to 40°–45° C. until sown.

3. Make loop dilutions in the melted agar and, as soon as the tubes are sown, cool them rapidly in an upright position.

Aerobic organisms grown in the upper part of the medium which contains a certain amount of air in solution, while the anaerobes multiply in the deeper layer.

Roux's Method. 1. Make a deep agar or gelatin stab or shake culture of the organism or substance to be studied.

2. Pour upon the surface of this medium a layer 1 to 2 cm. deep of a broth culture of a vigorous obligate aerobe as

B. subtilis, or an equal depth of liquefied agar or gelatin and inoculate this when solid with the aerobe.

The growth of the aerobe will use up all the oxygen that reaches it and will not allow any to pass through to the medium below, which will consequently remain in an anaerobic condition.

Giltner's H-tube Method. The use of Giltner's H-tube allows the anaerobe in a certain medium to be grown on one side of the H either as a stab culture or a streak, while the aerobe in the same or a different medium, liquid or solid, may be grown on the other side. Rubber stoppers, fitted to mouths of both tubes, are superimposed on cotton plugs. The aerobe soon exhausts the oxygen from the tube, allowing the anaerobes to develop.

This is the method recommended for determining the presence of and isolating *Bact. abortus* from infected mucous membranes and tissues. This organism when first isolated from tissues is a partial anaerobe, i.e., when an agar shake culture is made in an ordinary test tube the colonies develop in a *zone* about 0.5 cm. in width about 1.5 to 2 cm. below the surface of the agar.

By the use of the H tube, surface colonies of this organism may be readily obtained for study.

Novy Jar Method. This same principle may be applied by the use of separate tube or plate cultivations of anaerobes and aerobes in a Novy jar or similar apparatus; the aerobic organisms should be offered a large surface for growth in each case,

IV. DISPLACEMENT OF AIR BY INDIFFERENT GASES

The special tubes, flasks and jars adapted to cultivation of anaerobes in a vacuum are equally applicable in this method.

The gas generally employed is hydrogen. It is preferable to other gases not only because it is easily prepared, but that it has no injurious effects on the organisms.

A Kipp generator is connected up with three wash bottles, containing:

- (a) 10% lead acetate solution to remove H_2S ;
- (b) Silver nitrate solution to remove AsH_3 ;
- (c) 10% pyrogalllic acid solution made alkaline to remove any trace of oxygen, may be used to furnish hydrogen.

Hydrogen is most conveniently obtained by keeping a cylinder of the compressed gas in the laboratory. This gas generally contains about 99.6% hydrogen, the remaining 0.4% is mostly or entirely air, which represents 0.08% oxygen. The gas so kept requires no preliminary washing, but may be passed direct from the cylinder into the jar or flask.

Carbon dioxide is harmful to a large number of organisms, as is also coal gas. Nitrogen is satisfactory, but its method of preparation is so difficult that its use should be abandoned in practical bacteriology unless it can be obtained compressed in cylinders.

EXERCISE 2. THE EFFECT OF ANAEROBIC CONDITIONS UPON MICROORGANISMS FROM MANURE

Apparatus. Modeling clay; tubes of sterile gelatin; three sterile Petri dishes; three sterile deep-culture dishes (use top of Petri dish for cover); sterile 1 c.c. pipettes; sterile dilution flasks; six tubes of sterile agar; pyrogalllic acid; 10% solution of sodium hydrate; absorbent cotton.

Culture. Horse manure.

Method. 1. Plate the manure (1 gm. in 99 c.c. dilution flask) in duplicate in the Petri dishes and in the deep culture dishes, using dilutions 1 : 100, 1 : 10,000 and 1 : 1,000,000.

2. As soon as the agar is solid, invert the deep culture dishes containing the dilutions.

3. Place a *small* piece of absorbent cotton in the center of the cover. This must not touch the agar.

4. On the absorbent cotton, place 1 gm. of pyrogalllic

acid crystals; then place 10 c.c. of 10% NaOH in the cover of the dish. (The cotton prevents a too rapid reaction between the chemicals.)

5. Seal *at once* by packing the space between the cover and bottom air tight with modeling clay. Then mix the chemicals.

6. Place all six plates at room temperature.

Note. In the reaction which takes place between pyrogalllic acid and NaOH, oxygen is used and an anaerobic condition is established within the culture dish (exact reaction not known.)

7. Count the organisms after seven days. Estimate the number of different types of colonies developing under the varying conditions of air supply and note growth. Conclusions?

8. Compare your results with those of others and draw conclusions.

9. Make gelatin stabs of three or four of the predominant types of colonies on the anaerobic plates and cultivate anaerobically by Hesse's method. What types of organisms are these morphologically and culturally?

10. Are any types found on aerobic plates which are lacking on the anaerobic plates and vice versa?

What type of anaerobe is frequently found in horse manure?

11. When do anaerobic conditions exist in milk? In soil? Is this beneficial or otherwise in each case? What relation may there be between age of milk and type of colonies? Can this same relationship apply in the case of soil?

12. What other methods may be used for obtaining anaerobic conditions for microbial growth? Name the obligate anaerobes.

13. How is an organism isolated which is tolerant of an amount of oxygen less than that of the atmosphere, but will not grow under strictly anaerobic conditions?

14. State your results for the experiment in detail and

point out any conclusions that may be drawn. Mention any practical applications to be made.

REFERENCES

- EYRE: Bacteriological Technic, 2d Ed., pp. 236-247.
MARSHALL: Microbiology, pp. 93-98, 228-232, 306-331, 634-640.
BESSON: Practical Bacteriology, Microbiology and Serum Therapy, pp. 87-105.
GILTNER: Suggestions for partial anaerobic cultures. Science, n. s., Vol. XLI, No. 1061, p. 663.

EXERCISE 3. TO DEMONSTRATE THAT ACIDS ARE FORMED FROM CARBOHYDRATES BY BACTERIA

Apparatus. Three tubes of sterile litmus lactose agar; three tubes of sterile dextrose agar containing CaCO_3 ; sterile dilution flask (containing about 150 c.c. sterile salt solution); six sterile Petri dishes; sterile 1 c.c. pipettes.

Culture. Fresh milk culture of *Bact. lactis acidi*.

Method. 1. Place a *very small* loopful of the *Bact. lactis acidi* culture in the dilution flask. (Transfer from the white portion of the litmus milk culture.) Shake well.

2. Make three plates from each medium, using widely varying amounts, for example, 0.5 c.c., 0.1 c.c. and 1 drop. *Just before plating*, mix the CaCO_3 *well* with the agar (avoid air bubbles).

3. Place the plates (inverted) at room temperature.

4. Examine each daily after forty-eight hours. Note how each medium is changed by the growth of the colonies. Explain what has happened.

How is the object of the experiment demonstrated in the case of each medium?

5. Compare the size of colonies on the different media; also on each dilution of one medium; explain. Why are the colonies smaller on the thickly seeded plates?

6. Write the chemical equation with a specific enzyme for each change in the case of each medium.

7. Write the reaction involving the CaCO_3 .

8. State your results for the experiment in detail, draw any conclusions and point out any practical applications that may be made.

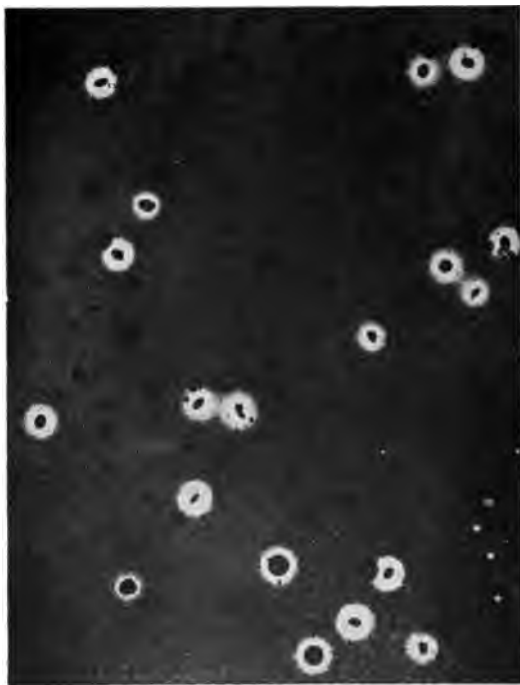


FIG. 43.—Solution of Calcium Carbonate by *Bact. lactis acidii*.
(Orig. Northrup.)

REFERENCES

- LÖHNIS, F.: Laboratory Methods in Agricultural Bacteriology, pp. 71-73.
VERNON: Intracellular Enzymes, p. 98.
EULER-POPE: General Chemistry of the Enzymes, pp. 31 and 58.
MARSHALL: Microbiology, pp. 96, 102, 107, 108, 306-312.
RAHN, O.: Tech. Bul. No. 10, Mich. Experiment Station, Fermenting Capacity of a Single Cell of *Bact. lactis acidii*, pp. 22-23.

EXERCISE 4. TO SHOW THAT ORGANIC ACIDS MAY SERVE AS A FOOD FOR SOME ORGANISMS

Apparatus. Two sterile 200 c.c. Erlenmeyer flasks; sterile 5 c.c. pipettes; 200 c.c. whey, soured by *Bact. lactis acidi*.

Cultures. *Oospora lactis*.

Mycoderma (pickle scum yeast).

Method. 1. Titrate the acid liquid and record the titre.

2. Place 100 c.c. in each flask.

3. Heat for one hour, cool and inoculate each flask with one organism.

4. Titrate every two days from the time growth shows until the reaction becomes constant. *Always use sterile pipettes for obtaining a sample for titration.*

5. Plot curves on the same paper, using the same zero point.

6. Did you place the organism in its natural habitat?

Will either of these organisms use another acid except that common to its habitat?

7. What is the chemical nature of this organic acid?

8. What has happened to the organic acid in question?

Write the chemical equation showing this action.

9. What type of enzyme is concerned in the change which takes place?

10. State your results in detail and point out any conclusions to be drawn. Point out any practical applications that may be made.

REFERENCES

LAFAR: Technical Mycology, English Ed., Vol. II, Part 2, pp. 417, 418, 452-455.

LASER: Biological Test for Butter. Zeitschrift für Hygiene (1891), Vol. X, p. 513.

MARSHALL: Microbiology, p. 111.

NORTHROP: Tech. Bul. No. 15, Michigan Experiment Station. The Influence of Certain Acid-destroying Yeasts upon Lactic Bacteria, pp. 5-7.

**EXERCISE 5. TO DEMONSTRATE THE VARIATION IN
FOOD REQUIREMENTS OF BACTERIA, AND THEIR
SELECTIVE POWER**

Apparatus. Two tubes of sterile fermented agar; two sterile Petri dishes; potassium phosphate, di-basic; asparagin; peptone; ammonium sulphate; sodium nitrate; dextrose; lactose; saccharose.

Culture. *B. prodigiosus*.

Method. 1. Melt the tubes of agar in the steam and, when cool but still liquid (about 40° C.), inoculate each heavily with *B. prodigiosus* and pour the plates. Allow to stand twenty-four hours at room temperature before proceeding. Is there any visible growth on the plate?

2. Mark on the bottom of each plate with drawing ink, dividing it into three equal sectors.

3. Use ink to indicate the places of chemicals, which should be deposited at the center of each plate and of each sector.

4. Use *very small* quantities of the chemicals and *be very careful not to scatter them* over the plate while conveying them to their proper places, otherwise the purpose of the experiment will be defeated.

5. Incubate at room temperature and examine the plate from day to day for growth.

Does the fermented agar support growth of itself? What explanation can you give for the action which occurs?

6. How is the variation in food requirements of *B. prodigiosus* shown? The selective action? Give another example of the demonstration of the selective action of bacteria. Which source of nitrogen is seemingly least available? Which most available? Why? Which carbohydrate is most easily digested? Which least? Why?

Beijerinck, knowing that agar in and of itself is a food for but very few microorganisms, reasoned that this sub-

SPLITTING OF CARBOHYDRATES INTO ALCOHOL 171

stance might be used for making solid synthetic media if it could be freed in some way from all traces of food materials. This he hoped to accomplish by allowing the agar to ferment spontaneously upon the addition of water.

In order that agar may not support microbial growth it must be allowed to ferment over a long period of time to exhaust every possible trace of food.

7. State your results in full, draw any conclusions that follow and point out the practical applications that may be made.

REFERENCES

MARSHALL: Microbiology, pp. 89-93, 98-100.

FISCHER, A.: Structure and Functions of Bacteria, p. 115.

EXERCISE 6. TO DEMONSTRATE THE SPLITTING OF CARBOHYDRATES INTO ALCOHOL AND CO₂

Apparatus. Clean 375 c.c. Erlenmeyer flask fitted with one-hole rubber stopper containing a bent glass tube plugged at the end with cotton; two calcium chloride tubes; potash bulb; calcium chloride (small granules); potassium hydroxide solution (1 part KOH, 2 parts H₂O); rubber tubing for connecting up apparatus; 400 c.c. fractional distillation flask; thermometer; 250 c.c. 5% saccharose bouillon.

Culture. *Sacch. cerevisiæ*.

Method. 1. Place the saccharose broth in the 375 c.c. flask, insert the rubber stopper and sterilize by the discontinuous method.

2. When sterile, inoculate with the yeast and connect the flask in "train" with a CaCl₂ tube (to remove moisture) a tared potash bulb (to take up CO₂) and a second CaCl₂ tube.

3. Place at 25° to 30° C. and allow to stand until no more gas evolves (about two weeks).

4. Test quantitatively for alcohol (distill off over 10 c.c.

of liquid, measure the distillate and determine its specific gravity*) and estimate percentage of yield.

5. Weigh the potash bulb to find the amount of CO_2 given off. Does it correspond to the yield of alcohol? Explain. Are your results according to theory?

6. Write the chemical equation for each change, giving the specific enzymes concerned in each reaction. What types of enzymes are concerned?

7. Would alcohol be formed in bouillon containing no sugar? In a 5% aqueous solution of sugar? Why?

What fermentable substances are present in ordinary meat bouillon?

8. State your results in full and draw any conclusions warranted. What practical applications may be made of the above?

REFERENCES

MARSHALL: Microbiology, pp. 135, 140-141.

LAFAR: Technical Mycology, Vol. II, Part II, pp. 473-481, 511-515.

HAWK: Physiological Chemistry, 4th Ed., pp. 255, 357.

EXERCISE 7. TO DEMONSTRATE THE NECESSITY OF NITROGEN IN SOME FORM FOR MICROBIAL GROWTH

Apparatus. Four tubes each of:

Ordinary broth (organic nitrogen, soluble albumins and proteins).

Dunham's solution (organic nitrogen, soluble peptone, no albumen).

Uschinsky's asparagin medium (organic nitrogen, protein-free).

Cohn's solution (inorganic nitrogen combined with organic acid).

* Table for determining per cent of alcohol from specific gravity in Sadtler's Industrial Organic Chemistry (1912), pp. 579-584.

Winogradski's medium for nitrate formation (inorganic nitrogen combined with an inorganic acid).

Winogradski's medium for symbiotic nitrogen-fixation (nitrogen-free).

Cultures. *B. subtilis*; *Ps. radiculicola*; *Aspergillus niger*; *Sacch. ellipsoideus*.

Method. 1. Inoculate heavily a tube of each medium with *Ps. radiculicola*. Proceed likewise with the three remaining organisms.

2. Record the growth at the end of five days. What conclusions may be drawn?

3. Compare the formulæ of the different media given above. *Organic nitrogen is present in the radical* NH_2 , *inorganic,* NH_4 .

4. What is the explanation for the growth of one organism and not another on a certain medium?

Do the organisms obtain their carbon from an organic or an inorganic compound in each case? Is organic or inorganic nitrogen the most available in each case? What is the value of such a medium? Why are other chemicals added besides the main nutrient?

Why is distilled water used in all these media? To what does the term "auxanography" refer?

5. Give your results in detail and draw any conclusions warranted. What practical applications may be made of the above?

REFERENCES

- MARSHALL: Microbiology, pp. 91-93, 99, 242, 362.
LAFAR: Technical Mycology, Vol. I, pp. 361, 468.
LÖHNIS: Laboratory Methods in Agricultural Bacteriology, p. 58.

EXERCISE 8. TO DEMONSTRATE THE PRODUCTION OF H_2S BY BACTERIA

Apparatus. Three tubes ordinary gelatin; tube ordinary agar; sterile Petri dish; lead carbonate, 0.1 gm.

Cultures. *B. coli communis*; *B. mycoides*; *B. mesentericus vulgatus*.

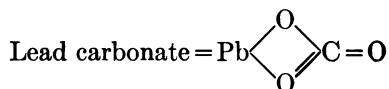
Method. 1. Make stabs of all organisms in gelatin and place these at a temperature not exceeding $20^{\circ} C$.

2. Melt a tube of agar and while hot add 0.1 gm. of lead carbonate to the tube and mix well by rolling it vigorously between the hands (avoid air bubbles).

3. Pour into the sterile Petri dish and when cold make a streak (2.5 cm. long and 3 cm. apart) of each organism on the plate in the order named. Invert and place at $25^{\circ} C$.

4. Examine the gelatin stabs from day to day for liquefaction; examine the plate culture at the same time. Note the action on lead carbonate (*Beijerinck's test*).

5. Write chemical equations for the action of sulphurated hydrogen on lead carbonate.



6. Is there any relation between the power of organisms to liquefy gelatin and to produce "lead-blackening" sulphur? Explain.

From what compounds is the H_2S produced in this experiment? What type of organisms can be detected by this test? Where do they occur in the largest numbers in nature?

Which of the ordinary laboratory media offer the greatest source from which this gas may be produced? Explain.

By what other means may H_2S production by bacteria be demonstrated?

7. Give your results in full. Draw any conclusions possible and point out any practical applications that may be made.

REFERENCES

- LAFAR: Technical Mycology, Vol. II., Part 2, pp. 558-560.
 MARSHALL: Microbiology, pp. 113-117.
 LÖHNIS: Laboratory Methods in Agricultural Bacteriology, pp. 42, 116.
 EYRE: Bacteriological Technic, 2d Ed., 1913, pp. 290-291.

EXERCISE 9. THE EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON MICROBIAL PIGMENT AND THEIR FORMATION

Apparatus. Six tubes of gelatin; six dextrose agar slants +15°; eight tubes of plain milk, sterile; hydrochloric acid; sodium hydroxide; chloroform; ether; benzol; carbon bisulphide; litmus paper; clean slide; small funnel.

Cultures. *Ps. pyocyanea*; *B. violaceus*; *B. prodigiosus*; *Sarcina lutea*; *Torula rosea*; *B. cyanogenus*; *Bact. lactis acid.*

Method. A. *Effect of temperature on pigment formation.*

1. Make two dextrose agar streak cultures of each organism.

2. Place the cultures in duplicate at 25° C. and 37° C.

3. Examine every day or so and at the end of a week record the degree of pigment formation by ++, +, +-, -. *Brightness of pigment formation* should be considered in all cases, *not the amount of growth.*

4. Where is the pigment seen macroscopically in each case? Explain.

Does temperature have any influence on pigment formation? Does this correspond in each case with that of the natural habitat of the organism?

How do these several pigments differ? Of what importance is pigment production?

B. *Relation of air to pigment formation.*

Make gelatin stabs of all organisms and keep at or below 20° C. Note the place of pigment formation. Explain.

C. Relation of light to pigment formation.

Make two streaks of *B. prodigiosus*. Place one in *bright sunlight*, keep the other in the dark. Explain the results.

D. Effect of chemicals on pigment.

1. To one of the brightest pigmented cultures of *B. prodigiosus*, add 10 c.c. of 95% alcohol and shake vigorously. Alcohol dissolves the pigment.

2. Pour off into a flask and allow to settle. Filter.

3. Divide the clear filtrate into four parts.

To one, add a drop or two of HCl; note the result and explain. To the second add a drop or two of NaOH; note the result and explain.

Place the third in *bright sunlight* and note what happens.

Place a few drops of the fourth portion on a clean slide and allow to evaporate slowly. Examine crystals under microscope and draw. What are these crystals? Explain.

E. Solubility of pigment.

1. Make five dextrose agar streak cultures of *B. prodigiosus* and, when *well pigmented*, try the solubility of the pigment in (a) water, (b) chloroform, (c) ether, (d) benzol, (e) carbon bisulphide. Results?

2. Are any of the different bacterial pigments formed, water-soluble? What is the simplest method for determining whether the pigment produced by an organism is water-soluble?

F. Blue milk and "bloody" milk.

1. Inoculate milk tubes as follows:

Organism.	Alone.	+ Bact. lactis acidi.
<i>Torula rosea</i>		
<i>B. cyanogenus</i>		
<i>B. prodigiosus</i>		
Control.....		

and keep at 25° C. along with uninoculated control. Observe daily.

2. At the end of seven days test the reaction of each. Is there any relation between the reaction and pigment production?

3. What conditions are conducive to the formation of red milk? of blue milk?

How would you describe and explain "bloody" milk as produced by microorganisms to anyone unfamiliar with the phenomenon? How differentiated from true bloody milk?

4. State all results in full. Draw any conclusions warranted and point out the practical applications that may be made.

REFERENCES

LAFAR: Technical Mycology, Vol. I, pp. 105-122.

THRESH: Examination of Waters and Water Supplies, 2d Edition, pp. 43-44.

STERNBERG: Textbook of Bacteriology, pp. 130-132.

CONN: Agricultural Bacteriology, pp. 156-157.

EXERCISE 10. TO ILLUSTRATE ONE OF THE PHYSICAL PRODUCTS OF METABOLISM

Apparatus. Three gelatin slants (20° alkaline, 3% salt); rubber stopper to fit one of the gelatin tubes.

Culture. *Ps. lucifera* or some actively phosphorescing organism.

Method. 1. Make a streak culture of the above organism upon each of the gelatin slants.

2. With one of the cultures, boil a rubber stopper and insert in place of the cotton plug.

3. Place the stoppered culture and a second one (cotton-plugged) at 20° C., the third cotton-plugged culture at 5°-10° C.

4. Examine in the light and in the dark after twenty-four and forty-eight hours. Compare (in the dark) the two cultures at 20° C.; if there is a *marked* difference, loosen the rubber stopper and note what happens.

5. If there is no immediate result from loosening the

stopper, replace the stopper with a sterile cotton plug and note both cultures after twenty-four hours. What occurs in either case?

6. Which is the better temperature for the growth of this organism? Can you suggest a reason why? What is the natural habitat of this type of organism? Of what importance are they?

What would you conclude regarding the respiration of phosphorescent bacteria? What term is applied to bacteria exhibiting this phenomenon? Of what importance is this phenomenon?

7. State your results in full, and draw any conclusions. What practical application of the above may be made?

REFERENCES

LAFAR: Technical Mycology, Vol. I, pp. 123-126.

MARSHALL: Microbiology, (1911), p. 129

FISCHER, A.: Structure and Functions of Bacteria (1900), pp. 63-64.

ENZYMES: CLASSIFICATIONS AND REACTIONS

Enzymes can be classified in several different ways:

I. According to their place of activity as *endo-enzymes* (intracellular) or *exo-enzymes* (extracellular);

II. According to the type of food substance acted upon, as *proteolytic* (protein-digesting), *lipolytic* (fat-digesting), enzymes attacking carbohydrates, etc.;

III. The most satisfactory and inclusive classification is that denoting the chemical reactions produced by the enzyme during its activity. Enzymes may thus be called:

1. *Hydrolytic*, the addition of one or more molecules of water to the molecule of the substance acted upon.

2. Enzymes producing *intramolecular changes*, i.e., *causing a rearrangement of the atoms within the molecule*. In a few cases these changes may be hydrolytic (*urease*) but a

number of the enzymes of this class causes this rearrangement, splitting the molecule *without the addition to or subtraction from any elements therein*.

3. *Oxidizing*, the addition of oxygen to (or the subtraction of hydrogen from) the molecule of the substance acted upon.

4. *Reducing*, the subtraction of oxygen from (or the addition of hydrogen to) the molecule of the substance acted upon. The reducing enzymes are the only class of enzymes in the above classification acting upon inorganic compounds; some organic compounds are also acted upon, viz., litmus, methylen blue, etc.

5. *Coagulating*, unknown processes accompanied by coagulation. Enzymes whose actions are not so well known are those producing syntheses, isomers, acting anaerobically, etc.

Note. Euler's suggestion that the names of enzymes be formed from the compound acted upon, by suffixing "-ase," will be adhered to in all subsequent study of enzymes, the suffix "-lytic" for the adjective, and the suffix "-ese" for synthesizing enzymes.

Bayliss has suggested the ending "-clastic" for the adjective, criticizing the ending "-lytic" because the definition of "electrolytic," which must be granted priority, implies action by the agent rather than upon the substance indicated by the term. He also questions the existence of Euler's "synthesizing enzymes."

CLASSIFICATION OF ENZYMES

I. Hydrolytic Enzymes of:

A. Carbohydrates, including Glucosides, *carbohydrases*—general term.

1. Polysaccharides. $(C_6H_{10}O_5)_x$.

a. Celluloses: *cellulases*—general term.

b. Hemicelluloses: *cytases*—general term.

c. Starches, insoluble and soluble: *amylases*, (ptyalin, diastase)—general term.

d. Glycogens: *glycogenases*—general term.

e. Dextrins: *dextrinases*—general term.

2. Disaccharides. $C_{12}H_{22}O_{11}$.
 - a. Saccharose: *sucrase* (invertase, invertin)—specific term.
 - b. Lactose: *lactase*—specific term.
 - c. Maltose: *maltase*—specific term.
 3. Glucosides: *glucosidases*—general term.
 - a. Amygdalin: *amygdalase* (emulsin, synaptase)—specific term.
 - b. Tannin (digallic acid): *tannase*—specific term.
 4. Pentoses: $(C_5H_{10}O_5)_x$.
 - a. Pectoses: *pectases*—general term.
 - B. Esters: *esterases*—general term.
 1. Fats: *lipases* (steapsin)—general term.
 - a. Stearin: *stearinase*—specific term.
 - C. Proteins: *proteinases* or *carbamases*—general term.
 1. Protein-digesting.
 - a. Proteins broken down to proteoses and peptones: *peptase* (pepsin or acid-proteinase)—general term.
 - b. Proteins broken down further to polypeptids and occasionally to α -amino acids with a trace of ammonia: *tryptase* (trypsin or alkali-proteinase)—general term.
 - c. Proteoses, peptones, polypeptids and protamins broken down completely to α -amino acids with a trace of ammonia: *ereptase* (erepsin, protease)—general term.
- II. Enzymes producing intramolecular changes, acting on:**
- A. Carbohydrates (d-hexoses) $C_6H_{12}O_6$ to form:
 1. Alcohol, ethyl and carbon dioxide: *zymase*—general term.
 - a. Dextrose: *dextro-zymase*—specific term.
 - b. Levulose: *levulo-zymase*—specific term.
 - c. Galactose: *galacto-zymase*—specific term.
 2. Lactic acid: *lactic acid bacteria zymase*—specific term.

B. Acid amides (urea): *amidases*—general term, to form:

1. Ammonium carbonate: *urease*—specific term.

III. Oxidizing enzymes: *oxidases*—general term, of:

A. Ethyl alcohol: *alcoholase* (alcoholoxidase, vinegar-oxidase)—specific term.

B. Organic acids:

1. Lactic acid: *lactacidase*—specific term.

2. Acetic acid: *acetacidase*—specific term.

C. Tyrosin: *tyrosinase*—specific term.

IV. Reducing enzymes: *reductases*—general term, of:

A. Hydrogen peroxide:

1. *Catalase*—specific term, free oxygen liberated.

2. *Peroxidase*—specific term, transference of oxygen.

B. Organic dyes to leuco-compounds.

1. Methylene blue, litmus, azolitmin, indigo, etc.: *methylene-blue reductase*, etc.—specific term.

2. Methylene blue in the presence of formaldehyde (Schardinger's reaction): *perhydridase*—specific term.

C. Sulphur to H_2S : *sulphur reductase*—specific term.

D. Nitrates to nitrites, nitrates to NH_3 , etc.: *nitrate-*, *nitrile-reductase*, etc.

V. Coagulating enzymes.

A. Protein-coagulating.

1. Casein:

a. Of cow's milk: *caseinase* (rennin, rennet, chymosin)—specific term.

b. Of human milk: *parachymosin*—specific term.

2. Fibrin of blood: *thrombase* (thrombin)—specific term.

B. Carbohydrate-coagulating.

1. Pectin: *pectinase*—specific term.

ENZYMIC REACTIONS OF WELL KNOWN FERMENTATION PROCESSES

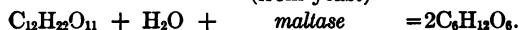
A. Beer or bread fermentation by *Saach. cerevisiae*.

I. starch + water + hydrolytic enzyme = maltose.



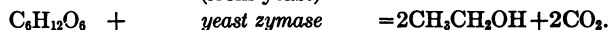
II. (a) maltose + water + hydrolytic enzyme = 2 mols. dextrose.

(from yeast)



III. dextrose + enzyme producing intra- = alcohol + carbon
molecular change dioxide.

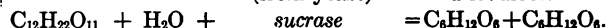
(from yeast)



This same yeast, and other yeasts also, can ferment saccharose (cane sugar) corresponding to II above, as follows:

II. (b) saccharose + water + hydrolytic enzyme = d-dextrose +

(from yeast) d-levulose.

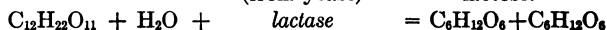


Both of these simple sugars can be fermented to alcohol and CO₂ according to III above.

Comparatively few yeasts can attack *lactose* (milk sugar), e.g., *Sacch. kefir* (p. 369, Marshall), *Sacch. fragilis*, *Sacch. tyricola*, etc. (See p. 106, Guilliermond's *Les Levures*.)

The reactions are similar to II (a) above, as follows:

(c) lactose + water + hydrolytic enzyme = d-dextrose + d-ga-
(from yeast) lactose.



Both simple sugars are changed to alcohol and CO₂ according to III above.

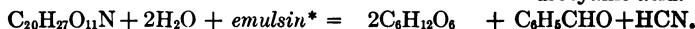
B. Glucoside decomposition, by molds, bacteria and yeasts.

General reaction,

glucoside + water + hydrolytic enzyme = sugar + aldehydes, acids, etc.

Specific reaction,

amygdalin + water + *emulsin* = dextrose + benzaldehyde + hydrocyanic acid.



* *Emulsin* is a mixture of four different enzymes.

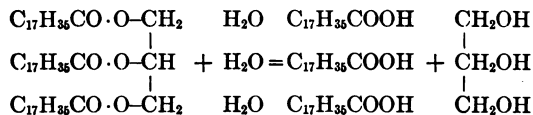
C. Fat decomposition, by a few molds, yeasts and bacteria.
Only microbial method of fat decomposition.

General reaction,

fat + water + hydrolytic enzyme = fatty acid + glycerin.

Specific reaction,

stearin + 3 mols. water + *stearinase* = 3 mols. stearic acid + glycerin.

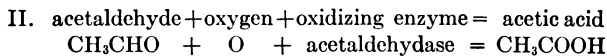
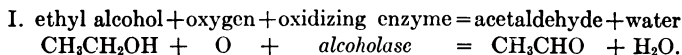


Lipases decompose more especially the natural fats, i.e., the glycerin esters of palmitic, stearic and oleic acids. Lipases from different sources differ markedly in reactions.

D. Vinegar fermentation.

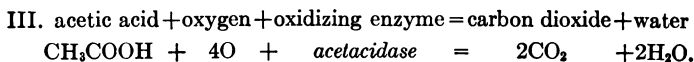
In order that this fermentation may take place, alcohol must be present in the nutrient solution, either added artificially or as a product of fermentation. In the latter case, reactions I, IIa and III, IIb and III, or IIc and III under A above must precede those of the vinegar fermentation.

Assuming that alcohol is present in the liquid in which the vinegar bacteria are growing, the reactions take place in two stages, as follows: (See p. 448, Marshall.)



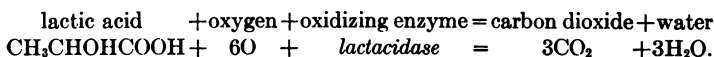
If there is not plenty of air present the oxidation may not become complete and small amounts of acetaldehyde may form, i.e., the reaction stops at the first stage.

If the initial percentage of alcohol is below 1 to 2% the vinegar bacteria will soon attack the acetic acid, oxidizing it completely to carbon dioxide and water, as follows:



This cannot take place, however, if above 10 to 12% acetic acid is present, as this amount is antiseptic to the vinegar bacteria. (See pp. 450-451, Marshall.)

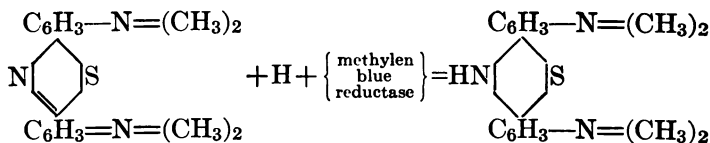
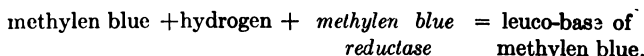
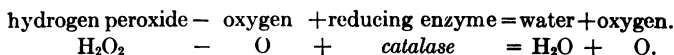
E. Organic acid decomposition, by acidophile organisms (organisms of the *Oidium* and *Mycoderma* type).



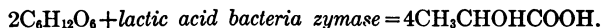
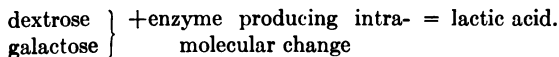
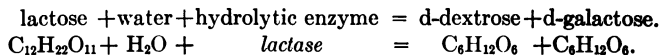
The destruction by oxidation of acetic acid by the acetic bacteria is given under *D* above.

Nearly all organic acids are decomposed in a similar manner, *by total combustion*.

F. Reactions of reductases.



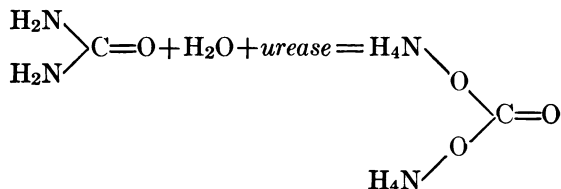
G. Lactic acid fermentation, produced in milk by *Bact. lactis acidi*.



Bact. lactis acidi will ferment a nutrient solution containing only a simple sugar, e.g., dextrose, the reaction then being according to the second equation.

H. Urea fermentation, by urea bacteria.

Urea + water + hydrolytic endo-enzyme = ammonium carbonate.
(producing intramolecular change)

**EXERCISE 11. A COMPARISON OF ACID AND RENNET CURDS**

Apparatus. Three 200 c.c. flasks containing 100 c.c. each of sterile skim milk; 200 c.c. *fresh* skim milk; small funnel; eighteen large test tubes; absorbent cotton; 10% lactic acid; 5% phenol.

Cultures. *B. prodigiosus*; *Bact. lactis acidi*; *B. megaterium*.

Method. 1. Inoculate flasks containing 100 c.c. sterile milk with *B. prodigiosus*, *Bact. lactis acidi* and *B. megaterium*.

2. Place about 30 c.c. fresh skim milk in a 200 c.c. flask.

3. Add 10% lactic acid *drop by drop*, shaking constantly.

4. When the *first finely divided* curd appears, titrate. What *degree* and *percent* of acid were necessary to curdle the milk?

5. Titrate fresh skim milk.

6. Prepare 150 c.c. of 0.5% phenol milk (by adding 15 c.c. of 5% phenol to 135 c.c. of milk and sterilize). Mix well and titrate again. Is the acidity of the milk increased perceptibly by the addition of phenol?

7. As soon as curd appears in inoculated flasks, titrate. Determine the *degree* and *percent* of acidity present.

8. Allow the cultures to develop several days until decided proteolysis is evident. Then titrate again and

filter each culture through absorbent cotton (a small piece in small funnel); 15 c.c. of each filtrate is necessary.

9. Mix the filtrate from each culture with phenol milk in the following proportions:

1. 0.5 c.c. filtrate + 9.5 c.c. phenol milk.
2. 1.0 c.c. filtrate + 9.0 c.c. phenol milk.
3. 2.0 c.c. filtrate + 8.0 c.c. phenol milk.
4. 3.0 c.c. filtrate + 7.0 c.c. phenol milk.
5. 4.0 c.c. filtrate + 6.0 c.c. phenol milk.
6. Heat 4 c.c. of filtrate only, in steam

for fifteen minutes. After cooling, add 6 c.c. phenol milk. Shake these mixtures well and incubate at 37° C.

10. Record the time necessary for coagulation in each case. Why do not all tubes change alike? Explain.

11. Can corrosive sublimate be used to replace phenol in this experiment? Explain.

What types of enzymes are concerned in these changes?

Are these intra- or extra-cellular in each case? Will the place of occurrence of the enzymes explain the action taking place in the different sets of tubes?

What enzymes produce each type of curd?

What are the differences between an acid and a rennet curd?

Which type is produced by each of the organisms used?

What effect has heat upon enzymes?

12. Give results in full and draw any conclusions permitted. Point out any practical applications of the above.

REFERENCES

- LAFAR: Technical Mycology, Vol. I, pp. 184-187.
BAYLISS: Nature of Enzymic Action, p. 37.
EULER: General Chemistry of the Enzymes, pp. 45-48, 58.
MARSHALL: Microbiology, pp. 139-141.
VERNON: Intracellular Enzymes, pp. 220-221.
COHNHEIM: Enzymes, pp. 29, 87-89.

EXERCISE 12. TO SHOW THE ACTION OF PROTEOLYTIC ENZYMES UPON GELATIN

Apparatus. Phenol, 0.5% solution (10 c.c. of 5.0% phenol + 90 c.c. distilled H₂O); water bath and thermometer; gelatin, 7 gms.; 15 tubes sterile gelatin; formalin; xylol; 5 sterile 1 c.c. pipettes; centimeter scale.

Cultures. *B. ramosus*; *B. fluorescens*; *B. subtilis*; *B. mycoides*; *B. prodigiosus*.

Method. 1. Make two gelatin stab cultures of each organism and when *nearly all* liquefied (2-5 days old) proceed with the experiment.

2. Dilute the 5% phenol to 0.5% as above, with distilled water.

3. Add 7 gms. gelatin, dissolving by heating not over 70° C. Neutralize carefully.

What is the source of acid in phenol gelatin? Why is the gelatin neutralized?

4. Select five test tubes having the same diameter. Fill each half full. Solidify in an upright position.

5. Shake each of the liquefied cultures with 3 to 4 c.c. of xylol.

6. After one hour, add 1 c.c. of the clear supernatant xylol solution of each culture to a tube each of solid phenol gelatin and of ordinary gelatin. With a blue pencil, mark the surface of the solid gelatin.

7. Examine the tubes daily. Is there any evidence of growth? Of liquefaction? If liquefaction is noted, measure its progress in millimeters.

8. Save the original cultures with which the xylol has been shaken. Is there any evidence of further growth?

9. If 1 c.c. of a liquefied gelatin culture of a liquefying organism were added to a tube of solid ordinary gelatin, what would happen? What would result if it were added to a tube of solid phenol gelatin? Explain.

10. What action does the xylol have? How can you

prove that xylol has this action? What other chemicals could be used in place of xylol?

11. What is the object of adding phenol to the gelatin? Would 5% phenol serve the same purpose? Give reason for answer. What other chemicals could be used in place of phenol? Why? What chemicals could not be used in place of phenol? Why? How else may *pure enzyme action* be demonstrated?

12. Add 5 drops of 40% formaldehyde (formalin) to each tube of the duplicate liquefied gelatin cultures and note whether they become solid again in a few days. *Explain the action.*

13. Give your results in full and draw any conclusions possible. What practical applications of the above may be made?

REFERENCES

- BAYLISS: Nature of Enzymic Action, p. 37.
MARSHALL, C. E.: Microbiology (1911), pp. 134, 138, 141.
EULER, HANS: General Chemistry of the Enzymes (1912), pp. 115-123.
VERNON, H. M.: Intracellular Enzymes (1909), pp. 215-220.

EXERCISE 13. TO SHOW THE ACTION OF PROTEOLYTIC ENZYMES UPON CASEIN

Apparatus. Five tubes of sterile milk; two tubes nutrient agar; sterile 5 c.c. pipettes; two sterile Petri dishes.

Cultures. *Bact. lactis acidi*; *B. ramosus*; *B. coli*; *B. violaceus*.

Method. 1. Warm the milk (40-45° C.).

2. Place 2 c.c. in each sterile Petri dish and pour one tube of melted agar upon it, mix thoroughly by carefully tilting.

3. When solid, make parallel streaks with *Bact. lactis acidi* and *B. ramosus* upon one and of *B. coli* and *B. violaceus* upon the other. Transfer cultures to litmus milk also.

4. Examine streak cultures *every* day for evidences of proteolysis. Make drawings and compare the rate of action of the different bacteria. Compare streak with milk cultures.

5. Is there any relation between the power of enzymes to liquefy gelatin and their ability to dissolve casein? What type of proteolytic enzyme dissolves casein?

6. Give your results in detail. Draw any conclusions which follow and point out any practical applications that may be made.

REFERENCES

MARSHALL, C. E.: Microbiology, pp. 138-139, 354.

HASTINGS: The action of various classes of bacteria on casein as shown by milk agar plates. Cent. f. Bakt., II Abt., Bd. 12 (1904) p. 590.

EXERCISE 14. TO SHOW THE ACTION OF ENZYMES UPON STARCH

Apparatus. Three sterile Petri dishes; three test tubes; soluble starch; three tubes sterile agar; Lugol's iodine solution.

Cultures. Soil for inoculation.

Method. 1. Place 0.1 gm. of soluble starch in each test tube, plug and sterilize in the hot air sterilizer.

2. To each tube of starch add one tube of melted agar.

3. When at the correct temperature (40°-45° C.) inoculate one tube with one loopful of soil. (State type used.) Inoculate the second from the first, etc., then plate all three dilutions.

4. When the colonies are well developed, pour iodine solution on the plate and note any clearing around the colonies. What does this indicate?

5. Examine microscopically different types of colonies attacking starch. Are they molds, yeasts, or bacteria? Which type predominates on your plates?

6. What enzymes are concerned? Give specific action. How is pure enzymic action demonstrated?

7. Write the theoretical chemical equation. What is soluble starch?

8. What is the value of such microbial action in soil? Where are starch-digesting microorganisms present in nature? Of what importance?

9. State results in full and draw any conclusions. Point out any practical applications of the above.

REFERENCES

- BAYLISS: Nature of Enzymic Action, pp. 25, 113.
 MARSHALL, C. E.: Microbiology (1911), pp. 90, 106, 248, 463, etc.
 EULER, HANS: General Chemistry of the Enzymes, pp. 13-15, *et al.*
 HAWK, PHILIP B.: Physiological Chemistry (1914), pp. 10, 48, 50, 61, 65.
 SADTLER, S. P.: Industrial Organic Chemistry, p. 186.
 LAFAR: Technical Mycology, Vol. II, Part 2, pp. 351-353.

EXERCISE 15. TO SHOW THE ACTION OF REDUCING ENZYMES

Apparatus. Petri dish; medium fine sand; sulphur; cake of Fleischmann's compressed yeast, *fresh* (obtain this yourself); small mortar and pestle; lead acetate paper.

Method. 1. Thoroughly grind the sulphur, sand and yeast cake in a small mortar.

2. Place the contents of the mortar in a covered dish with a piece of moistened lead acetate paper. What odors are noted?

3. What reaction is demonstrated by the lead acetate paper? What reactions are taking place? Give a chemical equation which will cover the final changes. May other enzymes be released from the yeast cells during the process of maceration? If so, what enzymes?

What names are applied to the specific enzyme acting on sulphur and to the class to which it belongs? Where does this action occur in nature?

This enzymic action was first observed in 1888 by a Frenchman, J. de Reypailhade, who found that the alcoholic extract of yeast would convert elementary sulphur into sulphuretted hydrogen.

4. Give all results in full and draw any conclusions permissible. What practical applications may be made of the above?

REFERENCES

- MARSHALL, C. E.: Microbiology, pp. 135, 142-143.
 LAFAR, F.: Technical Mycology, Vol. II, Part II, pp. 558-560.
 KRUSE, W.: Allgemeine Mikrobiologie, pp. 652-655.

EXERCISE 16. TO SHOW THE ACTION OF THE ENZYME CATALASE

Apparatus. Four fermentation tubes of nutrient broth (sterile); hydrogen peroxide (full strength).

Cultures. *B. coli*; *B. subtilis*; *B. mycoides*; *Bact. lactis acidi*.

Method. 1. Inoculate the fermentation tubes.

2. After growth is well started, add 1 c.c. of hydrogen peroxide to each tube and mix well.

3. After the tubes have stood for half an hour measure the gas formed. Compare your results with those of other students.

Note. If the bottle of H_2O_2 stands uncorked or in a warm place it decomposes very rapidly and the gas formed in the fermentation tubes will be much less than from a full strength solution.

4. What is the strength of commercial hydrogen peroxide?

Where else is catalase found? What is the type of action supposedly taking place? Write chemical equation showing the general reaction.

Have you ever observed the action of catalase produced in animal tissues? What is the difference between catalase and peroxidase?

5. State the results of your experiment in full and draw any conclusions permissible. Point out any practical applications that may be made.

REFERENCES

- BAYLISS: Nature of Enzymic Action, pp. 140-141.
EULER-POPE: General Chemistry of the Enzymes, pp. 65, 67-68.
VERNON: Intracellular Enzymes, pp. 127-132.
LÖHNIS, F.: Laboratory Methods in Agricultural Bacteriology, pp. 66-67, 79.
MARSHALL: Microbiology, pp. 135, 142-143.

EXERCISE 17. TO DEMONSTRATE THE OXIDIZING ENZYME OF VINEGAR BACTERIA

Apparatus. 200 c.c. fermented cider (or other fruit juice); sterile 375 c.c. Erlenmeyer flask; sterile 10 c.c. pipette; water bath; specific gravity bottle.

Culture. *Bact. aceti*.

Method. 1. Place the cider in a sterile flask and heat in a water bath at 60° C. for one hour. Cool quickly. What is this process called?

2. Determine the specific gravity of the fermented cider.

3. Inoculate with a pure culture of *Bact. aceti* and titrate every three days until the titre is constant.

4. Plot the curve showing and explain the direction which the curve takes. What is taking place? Enzyme? Chemical equation?

5. Determine the specific gravity of the solution at the last titration. How does this compare with specific gravity of cider vinegar of legal standard? What is the legal standard for vinegar in this state? Can you explain why all vinegar does not come up to the legal standard?

6. Is it practicable to use pure cultures for preparing vinegar? How do various species of vinegar bacteria differ from one another?

Under what conditions will acetic fermentation set in "spontaneously?"

What raw materials will give rise to a vinegar by a normal acetic fermentation?

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How does a scarcity of alcohol influence the amount of acid produced? An excess of alcohol?

How may vinegar be prepared artificially? How adulterated?

7. State the results of your experiment in detail and draw conclusions. Point out any practical applications that may be made.

REFERENCES

EULER-POPE: General Chemistry of the Enzymes, 60-61.

MARSHALL: Microbiology, pp. 135, 142, 448-451.

SADTLER: Industrial Organic Chemistry, pp. 266-272.

LAFAR: Technical Mycology, Vol. I, pp. 295-307.

Circular on "Vinegar" prepared by Bacteriological Laboratory, East Lansing, Mich.

EXERCISE 18. TO DEMONSTRATE THE NECESSITY OF AN ACTIVATOR FOR THE ENZYMIC ACTION OF RENNET (FROM CALF'S STOMACH)

Apparatus. Four clean 200 c.c. Erlenmeyer flasks; sweet skim milk (not over $+15^{\circ}$); rennet, fresh commercial; two 1 c.c. pipettes; sterile saturated solution of monobasic calcium phosphate ($\text{Ca}(\text{H}_2\text{PO}_4)_2 + \text{H}_2\text{O}$); water bath; thermometer.

Method. 1. Place about 150 c.c. of skim milk in each of two 200 c.c. flasks, plug these and sterilize them by the Tyndall method.

2. When ready to start the experiment, obtain 300 c.c. of fresh skim milk and place 150 c.c. of milk in each of the two remaining flasks.

3. Mark the fresh milk flasks Nos. 1 and 2; the sterilized milk flasks Nos. 3 and 4.

4. Place all four flasks in a water bath and heat the water to 35°C ., *not higher*. (*Steam cannot be substituted.*)

5. Mark the flasks as follows:

Flask No. 1 = unheated milk + rennet.

“ “ 2 = unheated milk + calcium phosphate + rennet.

“ “ 3 = heated (sterilized) milk + rennet.

“ “ 4 = heated (sterilized) milk + rennet + calcium phosphate.

6. Add 1 c.c. of the calcium phosphate solution to one flask of fresh milk and mix. (Flask No. 2).

7. To each flask of milk add a drop of rennet, shake quickly, replace the flask in the water bath and leave for ten to twenty minutes without disturbing.

8. Add 1 c.c. of calcium phosphate solution to flask No. 4. Shake quickly, return the flask to the water bath and leave for ten to twenty minutes without disturbing. Observe. If no curd appears, set the flasks at 37° C. and observe after about twenty-four hours. What is the explanation for the phenomena occurring in this flask?

9. Observe the milk in all flasks for curdling. Which flasks of milk curdled? Why?

10. What are the various synonyms of “rennet?”

What is the specific action of this enzyme?

What is the source of the enzyme used? How prepared?

What living organisms produce coagulating enzymes?

Does the rennet produced by various bacteria require soluble calcium salts for an activator? How would you determine this?

What is an activator? To what property of an activator is its action attributed? What are the different classes of activators? Do all enzymes require activators?

11. Give all results in full and draw any conclusions possible. What practical applications of the above may be made?

REFERENCES

LAFAR: Technical Mycology, Vol. I, pp. 185, 186.

BAYLISS: Nature of Enzymic Action, pp. 120-121, 132-133.

RICHMOND: Dairy Chemistry, p. 301.

EULER-POPE: General Chemistry of the Enzymes, pp. 94, 106-109.

EXERCISE 19. EFFECT OF CONCENTRATED SOLUTIONS UPON MICROORGANISMS

Apparatus. 750 c.c. nutrient broth; gelatin; salt; dextrose; saccharose; five 10 c.c. pipettes; 100 c.c. graduate.

Cultures. *Mycoderma*; *B. coli*; *M. varians*; *Sacch. cerevisiae*; *Penicillium*; *B. prodigiosus*.

Method. 1. Make up four tubes each of the following concentrations:

Electrolytes: sodium chloride, 5%, 10%, 15%, 20%, 25%.

Non-electrolytes: dextrose and saccharose, 30%, 45%, 60%, 75%, respectively.

Colloids: gelatin, 5%, 10%, 30%, 50%.

2. With the exception of the gelatin the separate weighing out for each concentration can be avoided by using the following method of mixing, with the stock solution containing 50% or 75% of the substance under study:

(a) Weigh out the correct quantity of material and place it in a 100 cc. graduate.

(b) Fill the graduate to the 100 c.c. mark with nutrient broth. Place the hand over the mouth of the graduate and shake until solution is complete. If necessary, fill to the mark again with broth. *For example:* Dissolve 25 g. of salt in about 90 c.c. of broth, fill the graduate to 100 c.c., to obtain a broth of which 100 c.c. contain 25 g. of salt. Mix this salt broth with common broth in the following proportions, by means of pipettes:

Salt broth.		Plain broth.	Salt content of mixture.
2 c.c.	+	8 c.c.	5%
4 c.c.	+	6 c.c.	10%
6 c.c.	+	4 c.c.	15%
8 c.c.	+	2 c.c.	20%
10 c.c.	+	0 c.c.	25%

Broth will give a precipitate after heating with salt, consequently each salt broth mixture has to be filtered separately after heating. What is this precipitated material?

Note 1. The stock solution of dextrose is best prepared by adding to 75 g. dextrose, 50 c.c. of broth, heating the mixture in the steam until dissolved and then making up to 100 c.c. with broth. The sugar solutions may have to be filtered also.

Note 2. As it is a difficult procedure to make up as high concentrations of gelatin as 30% and 50% with any degree of ease and accuracy, gelatin prepared according to the following procedure will serve to illustrate the point of the exercise.

With a blue pencil, mark the 10 c.c. level on each of sixteen tubes. To make up 5% gelatin, place 0.5 gm. gelatin in each of four tubes and make up to the 10 c.c. mark with broth. Proceed similarly with the remaining concentrations. After heating once, mix well with a sterile platinum loop.

Gelatin is practically the only colloid that can be obtained in solutions concentrated enough for this experiment (up to 70%). Great care must be taken to avoid the condensation of moisture on the sides of the test tubes or flask, because this moisture will reduce the concentration of the surface gelatin and thus cause incorrect data.

3. Sterilize the tubes by the intermittent method.

4. Inoculate *heavily* one tube of each concentration of the salt with *Mycoderma*, one with *B. coli* and one with *M. varians*. Inoculate tubes of each concentration of dextrose, saccharose and gelatin with *Penicillium*, *Sacch. cerevisiæ*, and *B. prodigiosus*, leaving a tube of each concentration uninoculated for control.

Note. The inoculation must be heavy, because experience teaches that a small inoculum is sometimes not sufficient to secure growth.

5. Note and tabulate the growth after seven days.

6. *Reinoculate* the lowest concentration of each set that does not show growth from the highest of the same set that does grow, e.g., if *Penicillium* grows at 45% dextrose but not at 60%, inoculate 60% from 45%. If it now grows, what is indicated? Is there not plenty of water and food material present? Explain your results.

Does the natural habitat or food requirements of each organism explain in any way the action occurring?

7. What is meant by osmotic pressure? Electrolyte? Colloid? What is known of the relative osmotic pressure

of electrolytes, non-electrolytes and colloids? How are these differences explained? Is the relative preserving power of these different substances according to the molecular weight theory? Explain.

Why is a large inoculum more apt to insure growth than a small inoculum?

Note. Salt-resisting organisms can be secured by plating in agar containing 10 to 15% of salt, from butter, brine pickles, salt pork, salt fish, and other salted food. Sugar-resisting organisms can be obtained similarly.

8. State the results of your experiment in full and draw conclusions. Point out any practical applications that may be made.

REFERENCES

MARSHALL: Microbiology, pp. 147-151.

FISCHER, A.: Structure and Functions of Bacteria, pp. 5, 8-9.

EXERCISE 20. THE EFFECT OF DESICCATION UPON BACTERIA

Apparatus. Four sterile cover-glasses; four sterile Esmarch dishes; potato knife; eighteen tubes of sterile broth.

Cultures. *B. violaceus* (non-spore-producing, non-slime-forming); slimy milk bacillus (non-spore-producing, slime-forming); *B. subtilis* (spore-producing, non-slime-forming); meat bacillus (spore-producing, slime-forming).

Method. 1. Using a platinum needle, smear one cover-glass thickly with a culture of *B. violaceus*, the second with the slimy milk bacillus, the third with the spore-former (*B. subtilis*) and the fourth with the spore and slime producing meat bacillus.

2. Place each of these cover-glasses in separate sterile Esmarch dishes and break each into five or six small pieces with a sterile potato knife.

3. Transfer a piece of each cover-glass to a tube of nutrient broth after 1, 3, 7, 14, etc., days.

Stop transferring when you find that there is no growth in the test tube last inoculated.

4. What influence has the physical condition of the substrate upon which the microorganisms are dried upon their longevity? Illustrate.

What dried cultures of microorganisms have been used with success commercially? Without success? What other methods may be employed to demonstrate the effect of desiccation on microorganisms?

5. State all results in full and draw any conclusions. Point out any possible practical applications.

REFERENCES

- MARSHALL: Microbiology, pp. 151-152, 280-283, 338-343, 367-369, 374-380, 428-429, 453-460.
SMITH, E. F.: Bacteria in Relation to Plant Diseases, Vol. I, pp. 70-71.
EYRE: Bacteriological Technic, 2d Ed., pp. 306-308.

EXERCISE 21. THE DETERMINATION OF THE OPTIMUM, MAXIMUM AND MINIMUM TEMPERATURE REQUIREMENTS FOR CERTAIN ORGANISMS

Apparatus. Sixteen tubes of dextrose broth.

Cultures. *Sacch. cerevisiæ*; *B. subtilis*; *Oospora lactis*; *Bact. aerogenes*.

Method. 1. Inoculate four tubes of dextrose broth with each organism.

2. Place one culture of each organism at each of the following temperatures: 5°, 25°, 37°, and 45°.

3. Note the growth as to vigor after twenty-four, forty-eight, seventy-two hours and seven days. Tabulate the data.

4. What is the natural habitat of each organism? Does this explain your results in any way?

What is the biological significance of the cardinal points of temperature? In what industries making use of microorganisms is the regulation of temperature especially important?

What inter-relations have the optimum, minimum and maximum temperature requirements of one species of microorganism?

What influence will the reaction of the medium have upon the extremes of temperature at which microorganisms will grow?

5. Discuss your results in detail and draw any conclusions permitted. Point out any practical applications.

REFERENCES

MARSHALL: Microbiology, pp. 153-157.

LAFAR: Technical Mycology, Vol. I, pp. 58-60.

JORDAN: General Bacteriology, 4th Ed., pp. 70-72.

EXERCISE 22. THE EFFECT OF FREEZING UPON SPORE-FORMING AND NON-SPORE-FORMING BACTERIA

Apparatus. Small ice dish; thermometer; three tubes each of sterile cider, sterile milk, sterile broth and sterile wort; coarse salt; ice.

Cultures. *Sacch. cerevisiæ*; *Bact. lactis acidi*; *B. megaterium*; *Aspergillus niger*.

Method. 1. Heavily inoculate the cider tubes with the yeast, the milk tubes with *Bact. lactis acidi*, the broth tubes with *B. megaterium* and the wort tubes with *Aspergillus niger*.

2. Incubate one set of cultures at 25° C.

3. Make sufficient freezing mixture of ice and salt to nearly fill the ice dish.

4. Carefully insert the two remaining sets of cultures in the freezing mixture and keep the freezing mixture at or below 0° C. for two hours.

5. Remove one set of tubes and incubate at 25° C.

6. Then place the ice dish containing the third set of cultures in the refrigerator (note the temperature).

7. Examine both sets of cultures at the end of twenty-four hours and forty-eight hours for growth.

8. Compare the three sets of cultures and note the variations from the normal type of growth. Tabulate your data.

9. Are all of these organisms *pecilothermic*? What are termed the cardinal points of temperature for micro-organisms? What is the lowest temperature at which growth, even of the feeblest kind, is possible? What term is applied to organisms which grow best at low temperatures?

10. Give all results and answers in full and draw any conclusions permissible. Point out the practical applications that may be made.

REFERENCES

FISCHER: Structure and Functions of Bacteria, pp. 73-75.

MARSHALL: Microbiology, pp. 154-155, 158-159, 199, 318, 395-401.

LAFAR: Technical Mycology, Vol. I, pp. 58-60.

EXERCISE 23. THE DETERMINATION OF THE THERMAL DEATH POINT OF A SPORE-FORMING AND A NON-SPORE-FORMING ORGANISM

Apparatus. Water bath; test-tube rack to fit bath; ring tripod; Bunsen burner; thermometer; thirteen test tubes of uniform diameter containing exactly 10 c.c. of broth; platinum loop 4 mm. in diameter; dish containing cold water (20° C. or below).

Cultures. Twenty-four to thirty-six hour broth cultures of *B. typhosus* and *B. mycoides*.

Method. 1. Examine the cultures for the presence or absence of spores.

2. Set up the water bath on the ring tripod, place only sufficient water in it to cover the medium in the test tubes and insert the test-tube rack.

3. Insert the thermometer into one of the test tubes of broth, passing it through the cotton plug.

4. After flaming the plugs, place all the remaining tubes

of broth in the rack in the water bath and heat slowly until the thermometer in the tube of broth registers 45° C.

5. Hold at this temperature for fifteen minutes.

Note. Slow heating is necessary in order that the respective temperatures may be held for the desired period of time.

6. *Without removing the tubes from the bath*, inoculate one tube of broth with a loopful of the broth culture of *B. typhosus*, a second with *B. mycoides*. Carefully mix the inoculum with the broth *without removing the tubes*. Mark each tube carefully.

7. Allow these inoculated tubes to remain in the water bath at 45° C. for ten minutes.

8. Remove and place immediately in cold water.

9. Incubate each organism at its optimum temperature after each trial.

10. Next, raise the temperature of the bath five degrees, i.e., to 50° C. and inoculate the tubes as before with *B. typhosus* and *B. mycoides*.

11. Keep the tubes at 50° C. for ten minutes, remove them from the bath, cool and incubate.

12. In the same manner expose the organism to the following temperatures, 55°, 60°, 65° and 70°, for a period of ten minutes each.

13. In all cases incubate seven days and record as the thermal death point (t. d. p.) the lowest temperature at which growth fails to appear.

14. What are the standard methods for the determination of the t. d. p.? What are the flaws in the above method? What different factors may influence the thermal death point of an organism?

Do all organisms possess the same t. d. p.? Explain.

15. Give data and results in full. Draw any conclusions that properly follow and point out any practical applications.

REFERENCES

- ROSENAU: Preventive Medicine and Hygiene, pp. 780-781.
JORDAN: General Bacteriology, 4th Ed., pp. 36-37, 72.
MARSHALL: Microbiology, pp. 159-161.
NOVY: Laboratory Work in Bacteriology, pp. 513-518.

EXERCISE 24. TO DETERMINE THE RELATIVE EFFECT OF MOIST AND DRY HEAT ON BACTERIA

Apparatus. Ten tubes of nutrient broth (large tubes); ten sterile Esmarch dishes; ten sterile (flamed) cover-glasses; autoclav; steam sterilizer; hot-air sterilizer.

Cultures. Agar culture of a spore-forming organism (having spores at the time).

Milk culture of slimy milk organism, non-spore-forming.

Method. 1. Make thick smears of each organism on five cover-glasses.

2. Place each cover-glass of the separate cultures in a sterile Esmarch dish and mark.

3. Place two Esmarch dishes of each culture in the hot-air sterilizer; heat to 120° C. and remove one dish of each culture after ten minutes at 120° C., the other two after thirty minutes.

4. Place two smears of each organism in the steam sterilizer; remove one of each after ten minutes, the two remaining after thirty minutes.

5. Place the two remaining Esmarch dishes in the autoclav and heat for ten minutes at 120° C.

6. When cool, transfer each of the cover-glasses to a tube of sterile broth; mark carefully.

7. Note in which tubes growth appears.

8. What is one of the most necessary factors for the prompt destruction of microorganisms by heat? Why?

Not considering moisture, what various conditions influence the destruction of microorganisms by heat? How are molds and yeasts influenced by moist and dry heat?

To what factors are the greater destructive powers of the autoclav due?

9. Give all data and results in full. Draw any conclusions possible and point out any practical applications.

REFERENCES

HITE, B. H., GIDDINGS, N. J., and WEAKLEY, CHAS. E.: The effect of pressure on certain microorganisms encountered in the preservation of fruits and vegetables. Bul. 146, W. Va. Univ. Agr'l Expt. Sta., 1914.

MARSHALL: Microbiology, pp. 159-161.

LAFAR: Technical Mycology, Vol. I, pp. 79-84.

FISCHER: Structure and Functions of Bacteria, pp. 75-77.

EXERCISE 25. TO DETERMINE THE EFFECT OF PASTEURIZATION UPON THE GROWTH OF MICRO-ORGANISMS

Apparatus. 300 c.c. each of milk (not sterile) and of some fermenting fruit juice; water bath; two thermometers; four sterile 200 c.c. Erlenmeyer flasks; twenty-four tubes of dextrose agar; dilution flasks; twenty-four sterile Petri dishes; sterile 1 c.c. and 10 c.c. pipettes.

Method. 1. Place 150 c.c. of milk in each of two 200 c.c. sterile Erlenmeyer flasks; do the same with the fruit juice.

2. Make three dilution plates each (1-100, 1-10,000, 1-1,000,000) from the milk and from the fruit juice in agar and incubate (inverted) at room temperature.

3. Place a flask of each nutrient liquid in the water bath (cold water) and heat rapidly to 75°-80° (thermometer in each flask), shaking the flasks frequently to obtain an even temperature throughout their contents.

4. Remove the flasks when the temperature reaches 80° C. and cool* them quickly.

5. Make dilution plates (1-10, 1-1,000, 1-10,000) in agar; mark each carefully. Place the flasks and plates at room temperature.

6. Place the other two flasks in the water bath (in cold

* It has been found by experiment that the *quick cooling* must take place through the temperatures 40°-36° C. in order to be most efficient in preventing further bacterial growth.

water) and heat *slowly* up to 60° (thermometer in each flask). Keep at 60°–65° for twenty minutes.

7. Remove the flasks from water bath and cool* quickly.

8. Make dilution plates, using the same dilutions as before, and place the flask and plates at room temperature, marking each carefully.

9. Watch daily for signs of growth in each medium.

10. Make plates from each flask after six days, determining the range of dilutions by consulting your former plates. Will the organisms have increased or decreased in this time? Why?

11. Compare the types of organisms on the plates before and just after pasteurizing and six days after pasteurizing. Examine each type microscopically. Of what does the predominant flora of each nutrient fluid consist before pasteurization? After pasteurization?

Note. The fruit juice may be saved for the experiment on metabiosis.

12. Count each set of plates and record the average number of microorganisms per c.c.

13. Plot the curve to show the destruction of microorganisms by pasteurization.

Compare the milk data with milk data and also the cider with cider.

14. Keep the original flasks for one or two weeks. If any marked changes occur, plate qualitatively and ascertain the type of organism causing the change.

15. How does the physical nature of the two nutrient substances influence their response to pasteurization? Give reasons for explanations offered.

What changes are brought about in milk by pasteurization? In cider or other fermenting fruit juice?

16. Give all data and results in full and draw any conclusions. Point out any practical applications that may be made.

* See note on page 203.

REFERENCES

- MARSHALL: Microbiology, pp. 319-320, 386-388.
RUSSELL and HASTINGS: Experimental Dairy Bacteriology, pp. 89-91.
LAFAR: Technical Mycology, Vol. II, Part I, pp. 142-144.

EXERCISE 26. TO ILLUSTRATE THE EFFECT OF THE REACTION OF THE NUTRIENT MEDIUM UPON MICROORGANISMS

Apparatus. One liter of ordinary broth (should be enough for three students); normal NaOH; normal acid; four sterile 1 c.c. pipettes; 10 c.c. pipettes; sterile test tubes.

Cultures. *B. prodigiosus* (broth culture); *B. subtilis* (broth culture); *Oospora lactis* (broth culture); *Torula rosea* (broth culture).

Method. 1. By adding normal acid or alkali produce in 100 c.c. portions of ordinary broth the following reactions: -40, -30, -20, -10, 0, +10, +20, +30, +40, +50 degrees Fuller's scale, and titrate after readjusting the reaction, as a check.

2. Tube, using 9.9 c.c. in each tube (*mark the tubes plainly*), and sterilize (refiltration may be necessary before tubing in some cases).

3. Using a sterile 1 c.c. pipette, inoculate one set (ten tubes) with 0.1 c.c. of the broth culture of each of the above organisms (four sets) and incubate the tubes at room temperature.

4. Examine the tubes as often as possible for the first twenty-four to thirty-six hours, and record the tube or tubes in which macroscopic growth is first visible. What do you conclude as to the effect of the reaction of the medium in these instances?

5. Examine the tubes every day for seven days. Tabulate your observation. Note the range of reaction in which each organism is capable of growing. Does this

range differ with different organisms? Explain the action occurring.

6. In each case inoculate heavily the first tube at either or both extremes, in which the organism fails to grow, from the tube just next it in series which shows growth. Does this freshly inoculated tube show signs of growth after twenty-four to forty-eight hours? Explain the action which occurs.

7. Which organisms are acidophiles?

What is the optimum, the minimum and the maximum reaction for each organism according to this experiment?

What factors not considered in this experiment might influence results?

How would you determine the exact optimum reaction of an organism?

8. Give all data and results in full and draw conclusions. Point out any practical applications.

REFERENCES

MARSHALL: Microbiology, pp. 173-180, 235-237.

EYRE: Bacteriological Technic, 2d Ed., pp. 305-306.

EXERCISE 27. TO DETERMINE THE INFLUENCE OF DIFFUSED LIGHT ON MOLDS

Apparatus. Sterile deep culture dish; tube of dextrose agar or gelatin; black paper.

Culture. *Rhizopus nigricans*.

Method. 1. Pour a tube of agar into a deep culture dish.

2. When solid, inoculate with *Rhizopus nigricans*.

3. Wrap the dish closely in black paper so that no light can penetrate.

4. Cut a hole 2 to 3 cm. in diameter in the top edge of the paper and place the dish in a north window so that only diffused light will enter the aperture.

5. Allow the dish to stand ten days, then examine it. How does diffused light influence this mold?

6. What is the term applied to this type of action? How are mold spores influenced by light? What influence does diffused light have on other microorganisms? Is it to be expected that other common molds, *Penicillium*, *Oospora*, *Aspergillus*, etc., would exhibit this same phenomenon?

7. Give all data and observations in detail. Draw any



FIG. 44.—Phototropism Exhibited by *Rhizopus nigricans*. The mold was grown on gelatin with diffused light coming from the right side. (From Marshall.)

conclusions that follow and point out any practical applications.

REFERENCES

- MARSHALL: Microbiology, pp. 162-165.
JORDAN: General Bacteriology, 4th Ed., p. 73.

EXERCISE 28. TO SHOW THE INFLUENCE OF DIRECT SUNLIGHT UPON THE GROWTH OF MICRO-ORGANISMS

Apparatus. Two tubes of sterile agar; two sterile Petri dishes; two sterile 1 c.c. pipettes; two tubes of sterile



FIG. 45.—Action of Direct Sunlight on Bacteria. These plates were heavily inoculated with *B. coli* and *B. prodigiosus*, respectively, and then were exposed bottom side up to the direct rays of the January sun for four hours. At the moment of exposure the figure 0, cut from black paper, was pasted to the plate, shading the bacteria underneath. After one, two and three hours the corresponding figures were pasted to the plates. The above picture was taken twenty-four hours after exposure, proving that three or four hours' exposure to direct sunlight weakens and may even kill bacteria. *B. prodigiosus* proved more sensitive than *B. coli*. (From Marshall.)

distilled water, salt solution or broth for dilution purposes; black paper; glue.

Cultures. *Ps. campestris*; *B. typhosus*.

Method. 1. Inoculate a tube of sterile liquid heavily with *Ps. campestris*. Mix the contents well.

2. Place 1 c.c. of this suspension in a sterile Petri dish and pour the plate.

3. Duplicate with the *B. typhosus* culture, placing the pipettes immediately after using in 1-1,000 HgCl₂.

4. Cut any design out of black paper and paste on the bottom of the Petri dish.

5. Place the dish bottom side up in direct sunlight for two hours.

6. Set the dish away in the dark at room temperature. Observe the growth and explain. Which organism is the more sensitive to sunlight? Conclusions?

Note. Heat *B. typhosus* plate 1 hour in steam before cleaning the Petri dish!

7. What theories have been advanced as to the mechanism of destruction by direct sunlight? Does sunlight have any effect on bacterial spores?

How are other forms of organisms affected by light?

What is phototaxis? Do bacteria ever exhibit this phenomenon?

Which portion of the spectrum is most active?

What relation does the wave-length of light rays bear to the activity of the rays?

How do diffuse light, electric or other forms of artificial light, X-rays, radium rays, etc., compare with direct sunlight as to their action on bacteria in general?

8. Give all data and state results in full. Draw any conclusions that follow and point out any practical applications.

REFERENCES

- MARSHALL, C. E.: Microbiology, pp. 162-163.
SMITH, ERWIN F.: Bacteria in Relation to Plant Diseases, Vol. I, p. 71; Vol. II, p. 324.
LAFAR, F.: Technical Mycology, Vol. I, pp. 60-63.

EXERCISE 29. DETERMINATION OF THE PHENOL COEFFICIENT OF SOME COMMON DISINFECTANTS

(Two students working together are required in this exercise.)

Apparatus. Copper water bath; test-tube rack for above bath, thirty-two test tubes of uniform size containing exactly 5 c.c. of sterile nutrient broth (use a graduated burette or a similar apparatus for filling tubes); eight clean dry test tubes of *uniform* size; several (4 or 5) platinum loops, of 4 mm. *inner* diameter; sterile 1 c.c. pipettes with fine point; three clean 5 c.c. pipettes; phenol, 5%; mercuric chloride, 1 : 500; small funnel; filter paper to fit funnel; sterile test tube; watch with second hand.

Culture. *B. typhosus*, twenty-four hour broth culture grown at 37° C.

Method. 1. Place the filter paper in the funnel, wrap in paper and sterilize in the hot air.

2. Filter the twenty-four-hour broth culture of *B. typhosus* into the sterile test tube. This is for the purpose of removing clumps of bacteria and any foreign matter. Funnel and filter paper are to be treated immediately after use with 1 : 1000 HgCl₂.

3. Regulate the water bath at 20° C. and keep at this temperature.

4. Mark the thirty-two test tubes, each containing exactly 5 c.c. of nutrient broth, with the name of the disinfectant, the dilution, and the time exposed, according to the following table. Then place the tubes, in order, in the rack in the water bath.

5. Mark each set of clean, dry test tubes carefully with the name of the disinfectant and the dilution to be added (see table on p. 212), and place in each, 5 c.c. of the dilution of the disinfectant as indicated on the labels. Keep in a test-tube rack at 20° C. *Work with one disinfectant at a time.*

N. B. Have the assistant carefully keep track of the exact time of all operations, to the second.

In actual practice determinations are made oftener than every five minutes, two and one-half minutes being the standard interval. This requires the most careful attention of both operator and assistant.

6. Using the 1 c.c. pipette, add 0.1 c.c. of the culture to one tube of each dilution of the disinfectant and *mix quickly* with a sharp rotary motion of the tube.

7. At the end of one minute from the time of each separate operation, make a loop transfer from the tube of each dilution of the disinfectant inoculated with the culture into the corresponding tube of broth in the water bath.

Note. The assistant takes the tubes from the water bath and hands them to the operator, then, after the operation of transferring, returns the inoculated broth tube to the water bath, sterilizes the needle and places it in the most handy position for the operator.

8. This operation is then repeated; working as quickly as possible, add 0.1 c.c. of the culture to the remaining tubes of the different dilutions of the disinfectant.

9. When, *in each case*, the culture has been exposed for exactly five minutes, ten minutes and fifteen minutes respectively to the action of the disinfectant, a loop transfer is to be made to the corresponding tube of broth.

10. When *all* transfers are made, place the broth cultures at 37° C. Examine after forty-eight hours for growth and record growth as + or -.

Note. The *phenol coefficient* of a disinfectant is the *ratio of the strength of the unknown disinfectant which will kill a filtered 24 hr. broth culture of B. typhosus in a certain length of time, to the strength of phenol which will accomplish the destruction in the same length of time, the dilution of phenol taken as 1.*

For example: The dilution of an unknown disinfectant required to kill *B. typhosus* in 7½ minutes was 1 : 550, and the dilution of phenol necessary to kill *B. typhosus* in the same time was 1 : 100. $550 \div 100 = 5.5$, the phenol coefficient of the unknown disinfectant. This means

that the unknown disinfectant undiluted is $5\frac{1}{2}$ times the strength of the undiluted phenol.*

11. Determine the approximate phenol coefficient of mercuric chloride according to the results of your experiment. How does this compare with results in literature?

12. What are some of the principal factors involved in the examination of disinfectants (pp. 12-20, Hyg. Lab. Bul. No. 82). How would each of these come into consideration in actual practice?

METHOD OF MAKING DILUTIONS OF DISINFECTANT FOR TEST

1 part of 5% phenol+1 part distilled water =2.5% phenol.

1 part of 5% phenol+4 parts distilled water=1.0% phenol.

1 part of 5% phenol+9 parts distilled water=0.5% phenol.

1 part of 1 : 500 HgCl_2 +1 part distilled water =1 : 1000 HgCl_2 .

1 part of 1 : 500 HgCl_2 +3 parts distilled water=1 : 2000 HgCl_2 .

1 part of 1 : 500 HgCl_2 +9 parts distilled water=1 : 5000 HgCl_2 .

METHOD OF RECORDING RESULTS

Disinfectant.	Time in minutes during which culture is exposed to action of disinfectant.			
	1 min.	5 min.	10 min.	15 min.
Phenol 5.0%.....				
Phenol 2.5%.....				
Phenol 1.0%.....				
Phenol 0.5%.....				
HgCl_2 1 : 500.....				
HgCl_2 1 : 1000.....				
HgCl_2 1 : 2000.....				
HgCl_2 1 : 5000.....				

13. Give data and results in full. Draw any conclusions that properly follow and point out any practical applications.

* In a large class it would be interesting to have determined the phenol coefficient of the chromic acid cleaning solution and of the 10% sodium hydroxide used for cleaning glassware, as each is recommended for immersing slides, cover-glasses, etc. contaminated with bacteria.

REFERENCES

- JOHN F. ANDERSON and THOMAS B. MCCLINTIC: I. Method of Standardizing Disinfectants with and without Organic Matter. Hygienic Laboratory Bul. No. 82 (1912), pp. 1-20, 34, 73.
- S. RIDEAL and E. K. RIDEAL: Some Remarks on the Rideal-Walker Test and the Rideal-Walker Method. Jour. of Infectious Diseases, Vol. X (1912), pp. 251-257.
- H. C. HAMILTON and T. OHNO: Standardization of Disinfectants. Reprint No. 45 (1913), from Research Laboratory of Parke, Davis and Co., pp. 451-458.
- The Bacteriological Standardization of Disinfectants. Reprint No. 65 (1914), from Research Laboratory of Parke, Davis and Co.
- M. L. HOLM and E. A. GARDNER: Formaldehyde Disinfection with Special Reference to the Comparative Value of Some of the Proprietary Products. Journal of Infectious Diseases, Vol. VII (1910), pp. 642, 643, 650, 658-659.
- MARSHALL: Microbiology, pp. 173-180.

EXERCISE 30. TO DETERMINE THE ACTION OF FORMALDEHYDE UPON THE MICROFLORA OF MILK

Apparatus. Fresh milk, skim or whole; 1% solution of formaldehyde; azolitmin solution; twelve sterile test tubes; H₂SO₄, concentrated *commercial*.

Method. 1. Make the following mixtures in sterile test tubes in *plain* milk, and *duplicate* in litmus milk (adding 2% azolitmin solution to the milk):

Milk.	Formaldehyde	Per cent Formaldehyde.
9.0 c.c. + 1 c.c. of 1%		= 0.1%
9.3 c.c. + 0.7 c.c. of 1%		= 0.07%
9.7 c.c. + 0.3 c.c. of 1%		= 0.03%
9.0 c.c. + 1 c.c. of 0.1%		= 0.01%
9.0 c.c. + 1 c.c. of 0.07%		= 0.007%
9.0 c.c. + 1 c.c. of 0.03%		= 0.003%

Place at room temperature.

2. Record the action in each tube, the time required for spoilage and the amount of formaldehyde necessary to preserve th

3. What is the lowest per cent of formaldehyde that has *inhibitive* action? That has *preservative* action? What terms are applied to these different percentages in each case?

4. Make a "ring" test for formaldehyde as follows: Add several drops of concentrated commercial H_2SO_4 to each tube of *plain* milk, allowing it to run down the side of the tube as in making an ordinary "ring" test. A violet coloration at the junction of the H_2SO_4 with the milk demonstrates the presence of formaldehyde in the milk. The presence of ferric chloride, an impurity in commercial sulphuric acid, is essential to this test.

5. Did all percentages of formaldehyde used give this test? Did all percentages which *preserved* give the test?

Is formaldehyde a desirable preservative for milk? Why? Are any chemicals more desirable for this purpose than formaldehyde?

What are the main uses of formaldehyde? What is paraformaldehyde? Its use?

6. State your results in full and draw any conclusions that follow. What practical applications may be made?

REFERENCES

MARSHALL: Microbiology, p. 179.

SAVAGE: Milk and Public Health, pp. 383-391.

HAWK: Practical Physiological Chemistry, 4th Ed., p. 239.

EXERCISE 31. TO ILLUSTRATE SYMBIOSIS

Apparatus. Sterile 5 c.c. pipettes; three sterile 200 c.c. Erlenmeyer flasks; 450 c.c. skim milk; three tubes of litmus milk (sterile).

Cultures. *Bact. lactis acidi*; *Oospora lactis*.

Method. 1. Place 150 c.c. milk in each flask and sterilize (Tyndall method).

2. Mark the flasks, *A*, *B* and *C*. Inoculate flask *A*

with *Bact. lactis acidi*, flask *B* with *Bact. lactis acidi* and *Oospora lactis*, and flask *C* with *Oospora lactis* alone.

3. Make ten titrations, titrating every two or three days (not oftener) and record the titrations. Tabulate the data.

4. Plot curves. How do you explain the direction these curves take?

5. At the end of the titrations, make loop transfers from each flask into litmus milk tubes and watch these carefully in the next twenty-four to forty-eight hours. Record the results.

6. Does the action in the flasks appear to be symbiotic? If so, how is it shown?

Is this symbiosis desirable or not? Explain.

What other well-known examples of symbiosis occur in nature? Give a reason for your statement.

7. Give all data and results in full. Draw any conclusions that follow and point out any practical operations.

REFERENCES

MARSHALL: Microbiology, pp. 181-182, 273-282, 323.

NORTHROP: The Influence of Certain Acid-destroying Yeasts upon Lactic Bacteria. Tech. Bul. No. 15, Mich. Expt. Sta., pp. 8-16, 32-34.

EXERCISE 32. TO ILLUSTRATE ONE OF THE PHASES OF MUTUAL RELATIONSHIP OF MICROORGANISMS

Apparatus. Sterile 5 c.c. pipette; 3 sterile 200 c.c. Erlenmeyer flasks; 450 c.c. sweet cider (that from pasteurization experiment may be used).

Cultures. *Sacch. ellipsoideus*, *Bact. aceti*.

Method. 1. Place 150 c.c. of sweet cider in each flask.

2. Determine and record the reaction of the cider, then heat the flasks thirty minutes in the steam.

3. Cool the flasks and inoculate flask *A* with *Sacch. ellipsoideus*.

4. Determine the weight *at once* and then every day until the weight becomes constant.
5. Inoculate flask *B* with *Bact. aceti* and flask *C* with both *Bact. aceti* and *Sacch. ellipsoideus*.
6. Titrate *B* and *C* every two days. Titrate flask *A* only at the end of the experiment.
7. Determine the amount of alcohol formed in flask *A*



FIG. 46. Antibiosis. This peculiarity of growth is the result of the inhibitive action of metabolic products diffused through the medium. (Orig. Northrup.)

by distilling the contents of the flask and determining the specific gravity of the distillate.

How much CO_2 was given off? Calculating from this amount, how many grams of sugar ($\text{C}_6\text{H}_{12}\text{O}_6$) were present in the flask? What percent sugar was this solution?

What was the theoretical amount of alcohol present?

8. Plot curves showing the acid formation in each case. Explain the direction which these curves take.

9. Explain the mutual action and the changes which occur.

10. What enzymes are responsible for each change? Write out the chemical equations for each change, giving enzyme concerned in each case.

Was the theoretical amount of alcohol changed into acetic acid? Give a reason for what really does happen.

What phase of mutual relationship is illustrated?

What is the classical example of this type of mutual relationship?

11. Give data and observations in full and draw conclusions. Point out any practical applications.

REFERENCES

MARSHALL: Microbiology, pp. 182-183, 448-458.

LAFAR: Technical Mycology, Vol. I, pp. 297-307; Vol. II, Part 2, pp. 473-481, 511-515.

EXERCISE 33. TO DEMONSTRATE THE EFFECT OF THE METABOLIC PRODUCTS OF *BACT. LACTIS ACIDI* ON ITS ACTIVITIES

Apparatus. Two sterile 200 c.c. flasks. 200 c.c. sweet skim milk; azolitmin solution; apparatus for titration; sterile dilution flasks; sterile Petri dishes; sterile 1 c.c. pipettes; sterile 5 c.c. pipettes; sterile 10 c.c. pipettes; ten to fifteen tubes of sterile litmus milk.

Culture. *Bact. lactis acidi* (twenty-four-hour culture).

(At least two weeks should be allowed for the completion of this experiment.)

Method. 1. Place 100 c.c. skim milk, $+10^{\circ}$ to $+15^{\circ}$ (record acidity before adding azolitmin), in each flask and sterilize by the Tyndall method.

2. Mark the flasks *A* and *B*.

3. Inoculate each flask with a loopful of a twenty-four-hour milk culture of *Bact. lactis acidi*. Mix well with a needle and plate dilutions 1-10, 1-100, 1 10,000 from flask

A for obtaining the initial number of *Bact. lactis acidi* introduced per c.c.

4. Continue as follows:

Flask A:

2d day, titrate and use dilutions 1-10,000, 1-100,000 and 1-1,000,000.

3d day, titrate and use dilutions 1-1,000,000, 1-10,000,000 and 1-100,000,000.

5th day, titrate and use dilutions 1-100,000, 1-1,000,000 and 1-10,000,000.

7th day, titrate and use dilutions 1-1,000, 1-10,000 and 1-100,000.

N. B. *Shake the flask of milk well each time before titrating and making dilutions.*

5. Titrate and plate every third day thereafter, until the acidity remains constant.

6. In flask B from day to day note in millimeters the extent of the re-oxidation of the azolitmin.

7. *Flask B.* Without disturbing the milk any more than necessary, make a loop transfer every day or so for 10 to 14 days from this flask into a tube of sterile litmus milk.

What occurs in each case? In what respects does flask B check up with flask A? Give explanations for similarity or dissimilarity of actions occurring.

8. Milk contains on an average about 4.5% lactose. Has this sugar been fermented entirely to lactic acid? Explain what really occurs.

9. What titre would milk containing 5% lactose have if this sugar were entirely changed to lactic acid?

Does any lactic-acid-producing organism approximate this reaction (in milk) at the height of its activity?

10. Give reasons for what occurs in each flask. What practical applications may this experiment have?

11. Tabulate your results and plot number and acidity curves. Explain these curves.

12. Draw any conclusions that follow from the above and point out any practical applications.

REFERENCES

- RAHN, O.: The Fermenting Capacity of a Single Cell of *Bact. lactis acidi*. Tech. Bul. No. 10, Mich. Exp. Sta., p. 25, *et al.*
MARSHALL: Microbiology, pp. 157, 308-312.

PART III

APPLIED MICROBIOLOGY

AIR MICROBIOLOGY

EXERCISE 1. QUANTITATIVE BACTERIAL ANALYSIS OF AIR

Apparatus. One carbon tube, dia. 15 mm.; cork stopper, perforated, to fit carbon tube; short piece of glass tubing bent at right angles; sand which has passed through a 150 mesh sieve; 8-liter aspirator bottle complete with rubber stoppers and glass tubing; sterile test tubes * containing 10 c.c. of sterile physiological salt solution; sterile 1 c.c. pipettes; four sterile Petri dishes; four tubes of sterile agar for plating; sterile agar slants; tubes of sterile broth; tubes of sterile litmus milk.

Method. 1. Prepare a sand filter aeroscope by placing a layer of cotton in the bottom of the carbon tube.

2. Upon this place 1 cm. of sand which has been run through a 150 mesh sieve.

3. Insert a cork stopper through which is passed a bent glass tube plugged at the outer end with cotton.

4. Sterilize the apparatus in hot air oven.

5. Place 8 liters of water in the aspirator bottle and mark the level of this amount of liquid.

6. Adjust the delivery tube so that it aspirates one liter of air per minute.

* For convenience in shaking the sample, it is recommended to use test tubes with aluminum screw caps, having cork packing.

7. Attach the aeroscope (lower end of carbon tube) to the aspirator so that the aspirated air will be filtered through the sand.

8. Remove the cotton plug from the upper end of the aeroscope and filter 8 liters of air in approximately eight minutes.

9. Using "aseptic" precautions, transfer as much sand as possible to one of the tubes of sterile salt solution.

10. Mix well by bumping the tube against the hand at least fifty times (do not wet the cotton plug).

11. Then, with a sterile 1 c.c. pipette, transfer 1 c.c. of the suspension to each of four Petri dishes and pour plates.

12. Incubate two plates at 37° C. for two days, and the remaining two plates at room temperature for five days.

13. Count at the end of these respective periods and determine the number of bacteria per liter. How do your counts compare with air counts obtained by other students? from other data? (See Marshall's Microbiology, p. 789.)

Make separate counts of molds and identify them as far as possible.

14. Make sub-cultures of different types on agar and study their cultural characteristics on this medium.

15. Transfer these cultures to tubes of broth and litmus milk and note their action on these media. Draw conclusions from these results.

16. What morphological types are found? Are any of the types of bacteria present constantly found in air? What are the sources of microorganisms in the air?

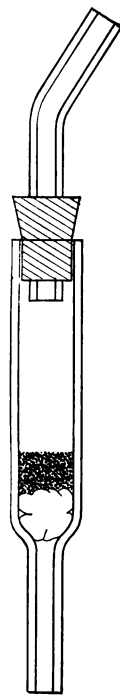


FIG. 47.—Modified Standard Aeroscope. (Ruehle and Kulp.)

Are any of the types isolated related to pathogenic forms? May pathogenic bacteria be isolated from air? If so, under what circumstances?

How do microorganisms enter the air? What types of microorganisms are most apt to be present in air? What is the explanation for this?

What other methods may be employed for obtaining quantitatively the bacteria in the air?

Of what importance is the quantitative or qualitative determination of microorganisms in air?

17. Give data and results in full and draw any conclusions permitted. Point out any practical applications of the above.

REFERENCES

- RUEHLE, G. L. A. and KULP, W. L.: Germ content of stable air and its effect upon the germ content of milk, *Bul. 409, N. Y. Expt. Sta.*, 1915.
- MARSHALL: *Microbiology* (1911), pp. 185-191.
- EYRE: *Bacteriological Technic*: 2d Ed. (1913), pp. 468-470.
- BESSON: *Practical Bacteriology, Microbiology and Serum Therapy*, transl. by Hutchens (1913), pp. 862-867.
- CHAPIN, C. V.: The air as a vehicle of infection. *Jour. Amer. Med. Ass'n.*, Vol. LXII, pp. 423-430 (1914).
- WINSLOW, C. E. A.: Bacteriology of air and its sanitary significance. *Cent. f. Bakt. Abt. II. Bd.*, 42, p. 71 (1914).
- WINSLOW, C. E. A. and BROWN, W. W.: The microbic content of indoor and outdoor air. *Mo. Weather Rev.*, Vol. XLII (1914), pp. 452-453. *Abst. in Exp't. Sta. Record*, Vol. XXXII, No. 3 (1915), p. 211.

WATER AND SEWAGE MICROBIOLOGY

**EXERCISE 1. BACTERIOLOGICAL ANALYSIS OF WATER
FROM A SOURCE NOT SUSPECTED OF SEWAGE
CONTAMINATION**

Apparatus. Sterile 500 c.c. flask for collecting water sample; litmus lactose agar shake; twelve tubes of litmus lactose agar; twelve salt-free gelatin tubes; two litmus lactose bile fermentation tubes; six agar slants; six tubes of sterile broth; six tubes of Dunham's solution; six tubes of nitrate peptone solution; six dextrose fermentation tubes; six tubes of litmus milk; twelve sterile Petri dishes; sterile 100 c.c. volumetric pipette; sterile 1 c.c. and 5 c.c. pipettes; record sheet for recording data obtained; record sheet for recording pure cultures isolated; water sample.

Cultures. *B. coli*.

Water from the local water system should be used for the experiment. This method can be used also for water from deep wells, springs, etc.

Method. 1. Flush the water pipes thoroughly by allowing the water to run, or by pumping, at least thirty minutes.

2. Hold the collection flask, mouth downwards, remove the plug and still holding in this inverted position, wash the mouth off with the running water, then fill quickly and replace the plug. *The plug must not be laid down during this process.*

3. The sample must be analyzed *at once*. In routine work, if this is not practicable, place the sample on ice and analyze *as soon as possible*. Samples kept at 10° C. or less should never be left over a maximum of six hours before analysis.

4. Plate immediately in duplicate, 1 c.c., 0.5 c.c. and 0.1 c.c. of the sample *direct* in litmus lactose agar and in gelatin (6 plates each). (If sewage contamination is sus-

pected the sample must be diluted. Use distilled water for dilution flasks.)

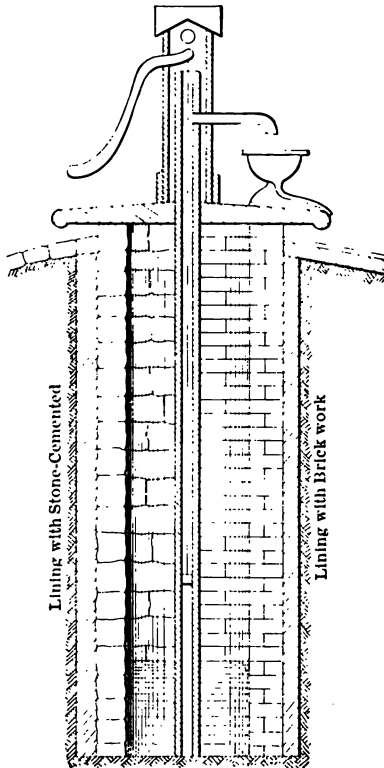


FIG. 48.

FIG. 48.—A Model Dug Well Constructed to Avoid Microbial Contamination of Water. (From Gerhard's Sanitation, Water Supply and Sewage Disposal of Country Houses.)

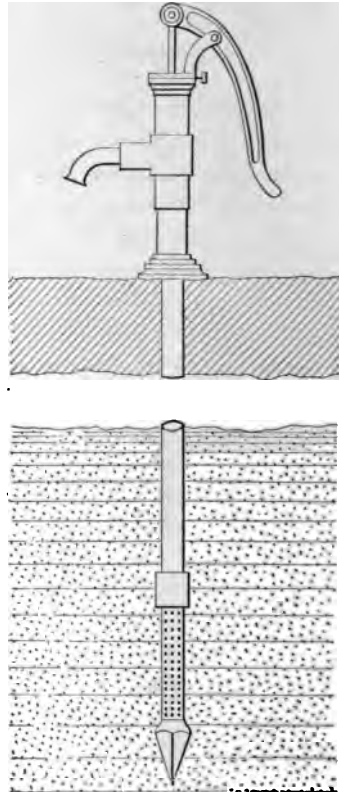


FIG. 49.

FIG. 49.—A Shallow Driven or Tube Well. (From Gerhard.)

5. To the melted agar shake (at 45° C.), add 100 c.c. of the sample, using the volumetric pipette, and shake well

to mix the sample thoroughly with the medium. Avoid shaking so violently as to produce gas bubbles.

6. Using a sterile 5 c.c. pipette, add 5 c.c. of the water sample to each litmus lactose bile fermentation tube.

7. Incubate the gelatin plates (*cover-side up*) in a cool place.

8. Incubate the agar plates (*cover-side down*), the agar shake and the fermentation tubes at 37° C.

9. Make note of the time of day these are placed in the incubator. All cultures placed at 37° C. *must be examined within twenty-four hours*. Types of colon-like organisms if present, may be quite easily recognized within twenty-four hours by the type and reaction of the colony on the agar plate, by the fermentation test, and by acid and gas formation in the agar shake.

10. Examine the agar plates after eighteen to twenty-four hours incubation.

11. If acid colonies are present, make morphological determinations (hanging drop) for their similarity to *B. coli*.

12. If these characteristics are positive, transfer five different colonies to agar slants.

13. Agar shake and fermentation tubes *must be examined in eighteen to twenty-four hours* also for gas and acid production.

14. If gas is present in either, and *no acid colonies have appeared on the original plates*, make dilution plates in litmus lactose agar in order to isolate the acid and gas producing organisms.

15. Transfer five different *acid colonies* which show a morphology similar to that of *B. coli* to agar slants.

16. At the end of forty-eight hours, remove the agar plates from the incubator, make counts, record the types of colonies present and the number of each type.

17. Transfer each type of colony not previously isolated, to an agar slant.

18. After all agar slant cultures have grown sufficiently (twenty-four hours at least), make sub-cultures from each pure culture into litmus milk, gelatin stab, nutrient broth, dextrose fermentation tube, Dunham's peptone solution and nitrate peptone solution for corroborative tests, and record the characteristics of growth according to the descriptive chart of the Society of American Bacteriologists, on the form on p. 235.

19. Determine to which group of water organisms each pure culture belongs. (Consult the table in Savage's Bacteriological Examination of Water Supplies, pp. 192-193.)

20. Compare pure cultures isolated from acid colonies with a pure culture of *B. coli* in each case.

21. Keep the plates at room temperature after forty-eight hours of incubation and count at the end of seven days. Record the counts as above.

22. Keep the agar shake and fermentation tubes for seven days and record any changes that take place.

23. Examine gelatin plates after forty-eight hours and then every day or so for seven days.

24. Count before the liquefying colonies get so numerous or large as to render counting difficult.

25. Record the total number of organisms per cubic centimeter; also the proportion of the liquefying to the non-liquefying organisms. Deep-well water should contain none or but very few liquefying organisms. Why?

26. If liquefying organisms are present in large proportion or in great numbers on the plates from deep-well water, re-plate from the same source (not from the original sample) to determine whether the liquefying colonies came from the original sample or from some fault of technic.

27. Fish each type of colony and determine to which group of water organisms it belongs. Are the same organisms found on gelatin as on agar plates?

28. Record and compare the number and types of organ-

isms developing on the agar and gelatin plates. Explain why your results vary on different media.

29. Does one medium seem more favorable to the development of a larger number of organisms? If so, which? Give reasons for answers.

30. If the gelatin count is less than 100 organisms per cubic centimeter the water is good, 200 per cubic centimeter will pass but if many more, i.e., 500, 1000 or over, the water is suspicious and effort should be made to determine the presence of *B. coli*.

31. Read the statements on pp. 41-43 and 62-64, et al, in Prescott and Winslow's Elements of Water Bacteriology and compare with those on p. 77, Standard Methods of Water Analysis. After experiments 1 and 2 are completed, draw your own conclusions from the above and give reasons for statements you make.

32. Each student must know the morphological and cultural characteristics of *B. coli*, *Bact. aerogenes* and *B. typhosus*.

Read about and comment on the methods used in other countries, giving references to the literature read.

33. What other methods are employed to determine the potability of water? Discuss these.

REFERENCES

- Standard Methods of Water Analysis, American Public Health Association (1913), pp. 77-80, 88-96, *et al*.
 PRESCOTT and WINSLOW: Elements of Water Bacteriology, 3d Ed., pp. 1-51, 215-228.
 MARSHALL: Microbiology, pp. 192-204.
 SAVAGE: Bacteriological Examination of Water Supplies (1906), pp. 192-193, 194-264.
 THRESH: Examination of Waters and Water Supplies, 2d Ed. (1913), pp. 184-271, 488-524.
 DON and CHISHOLM: Modern Methods of Water Purification (1911), pp. 260-271.

EXERCISE 2. BACTERIOLOGICAL ANALYSIS OF WATER SUSPECTED OF SEWAGE OR OTHER POLLUTION

Apparatus. Sterile 500 c.c. flask for collecting sample; litmus lactose agar shake; six litmus lactose agar tubes for making plates; ten litmus lactose (or ordinary) agar slants for pure cultures isolated; six salt-free gelatin tubes for plates; ten tubes of gelatin for pure cultures isolated; two litmus lactose bile fermentation tubes (p. 122, Prescott and Winslow); ten tubes of litmus milk; ten tubes of Dunham's peptone solution; ten tubes sterile esculin bile for *B. coli* test (p. 129, P. and W.); ten tubes of nitrate peptone solution; ten each fermentation tubes of dextrose, lactose and saccharose broth; 99 c.c. dilution flasks; twelve sterile Petri dishes; sterile 100 c.c. volumetric pipette; sterile 1 c.c. and 5 c.c. pipettes; sample of water or sewage from source indicated by instructor; record sheet for recording data; record sheet for pure cultures isolated.

Cultures. *B. coli*, *Bact. aerogenes*, *B. typhosus*.

Method. Water for this experiment may be obtained from a lake, river, etc., just below a sewer outlet, or from a surface well, etc., the source will be designated by instructor.

1. Collect the sample in the sterile 500 c.c. flask, using all precautions as with an unpolluted water sample. (See Exercise 1.)

2. This sample must be analyzed *at once*.

3. Using a wide range of dilutions, plate immediately in litmus lactose agar and in salt-free gelatin, making six dilution plates in each medium.

4. If the water is not suspected of great pollution, 0.1 c.c. of the sample may be plated directly, using low dilutions for the remaining five plates.

5. If pure sewage is to be plated, use dilutions 1 : 100, 1 : 1000, 1 : 10,000, 1 : 100,000, 1 : 1,000,000 and 1 : 10,000,000.

6. Make an agar shake as in Exercise 1 and inoculate lactose bile fermentation tubes with 0.1 c.c. direct and 1 c.c. of the 1 : 100 dilution of the sample respectively (or greater quantities if only a small amount of pollution is suspected).

7. Incubate the agar shake, fermentation tubes and agar plates at 37° C. Place gelatin plates at 15° to 20° C.

8. Make note of the hour at which the cultures are placed at 37° C. *Examine these within twenty-four hours* for indications of the presence of the colon-type of organisms.

9. Examine and count

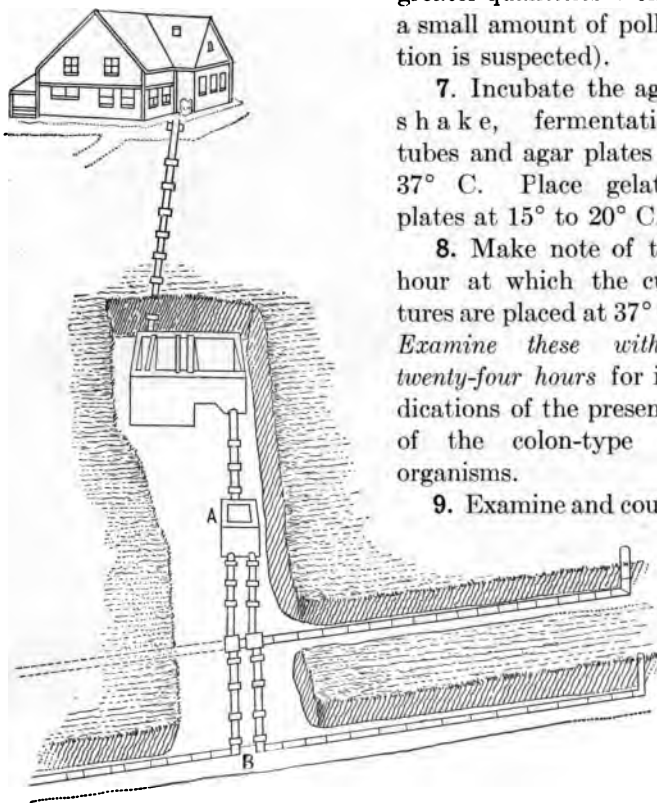


FIG. 50.—General Ground Plan of Individual House Sewer System Showing Exit from House, Anaerobic Tank, (A) Switch, and (B) Subsurface Irrigation Tile.

the agar plates after eighteen to twenty-four hours incubation at 37° C. (Keep at room temperature after forty-eight hours.)

10. Fish acid colonies, placing them on agar slants, and incubate at 37° C. for twenty-four hours.

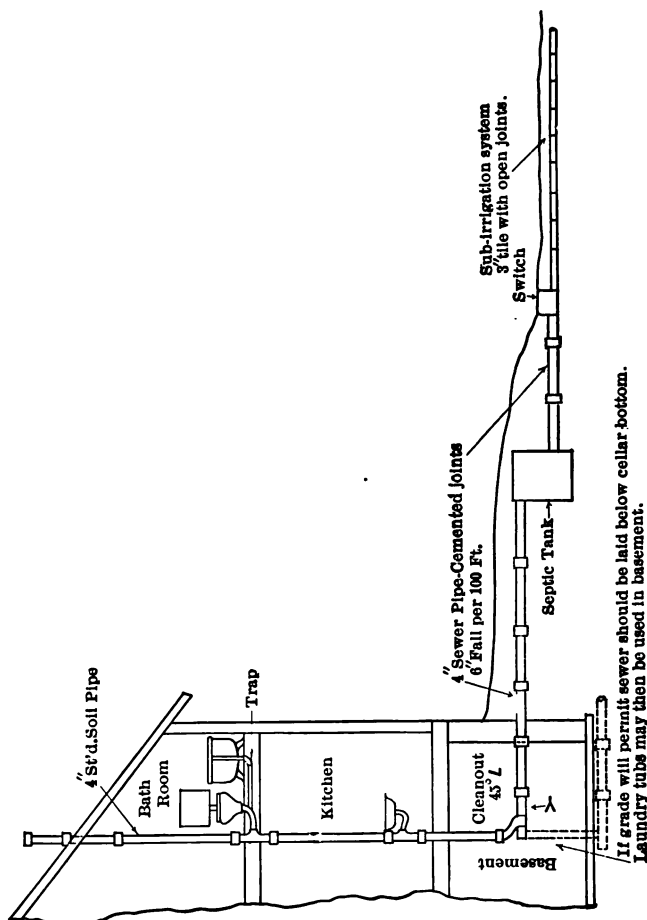


Fig. 51.—Sectional View of Same Sewer System.

11. Examine each colony microscopically in a hanging drop. If characteristic of *B. coli*, make sub-cultures in litmus milk, gelatin (stab), Dunham's solution, nitrate

peptone solution, dextrose, lactose and saccharose fermentation tubes, and esculin bile, for identification.

12. Test the gas for H and CO₂ and record the ratio.

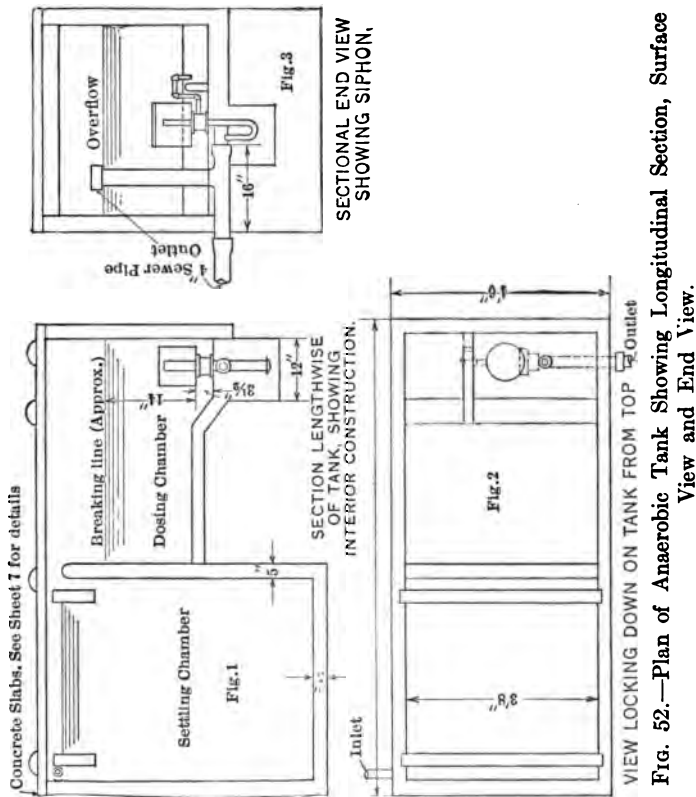


Fig. 52.—Plan of Anaerobic Tank Showing Longitudinal Section, Surface View and End View.

13. Transfer *B. coli*, *Bact. aerogenes* and *B. typhosus* to the same media and compare their cultural characteristics with those of organisms isolated.

14. Determine whether the organisms are Gram-positive or -negative.

15. Examine gelatin plates after two days and then every few days for seven days.

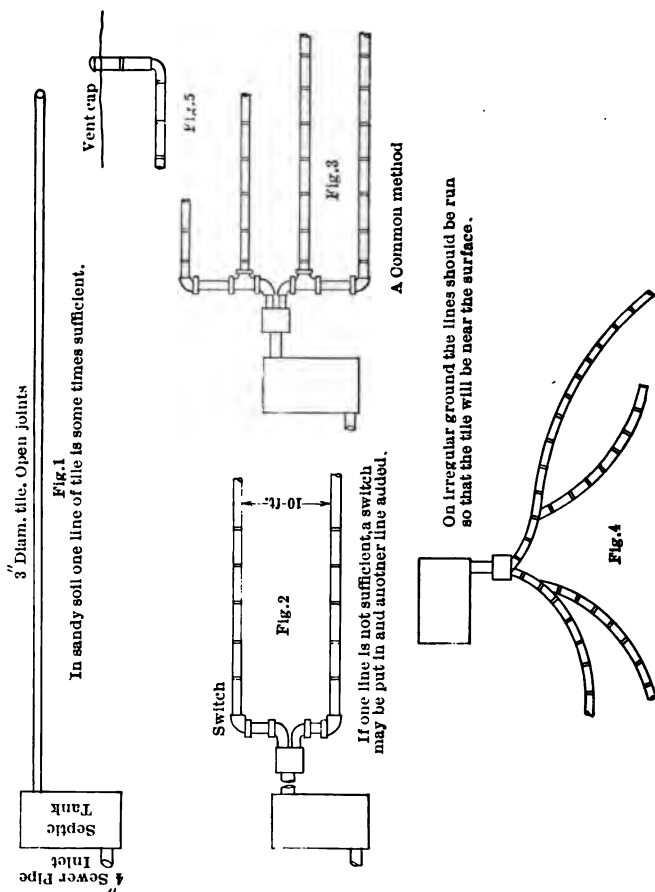


FIG. 53.—Details of Sub-surface Disposition of Effluent from Anaerobic Tank.
(Figs. 50-53 from material for public distribution from Farm Mechanics Department,
Mich. Ag. College, O. E. Robey.)

16. Count before the liquefying organisms get so numerous or large as to render counting difficult.

17. Estimate the ratio of liquefying to non-liquefying

organisms. What is the significance in sanitary water analysis of a large number of liquefying organisms?

18. If acid colonies are not present in twenty-four to forty-eight hours on litmus lactose agar plates, and acid and gas are evident within this time either in the agar shake or lactose bile fermentation tubes, make dilution plates from either of these in litmus lactose agar, incubate at 37° C. for twenty-four hours and fish acid colonies, placing them on the different media for differentiating *B. coli*.

19. Compare the cultures isolated with the pure culture of *B. coli* in every case. Also make comparisons with *Bact. aerogenes* and *B. typhosus*.

20. Isolate several different colonies and record the cultural, etc., characteristics of each organism on the record sheet furnished. Were any of the types of organisms in this sample present in the first sample? Would you expect to find this the case? Give reason.

21. The data and conclusions in the above should be given in detail. Point out also any practical applications.

REFERENCES

- Standard Methods of Water Analysis, 1913 edition, pp. 79-82, 87-88, 92, 95-102.
- PRESCOTT and WINSLOW: Water Bacteriology, 3d. ed., pp. 61-201, 228-265.
- SAVAGE: Bacteriological Examination of Water Supplies, pp. 27-69.
- MARSHALL: Microbiology, pp. 97, 108, 162, 182, 204, 212, 221, 323-324.

BACTERIOLOGICAL WATER ANALYSIS

Sample No. Date 19...
 Name of sender
 Address
 Source of water
 Surroundings
 Temperature
 Appearance
 Odor
 Remarks

Age.	Agar Shake.	Lactose Bile.		
		Per cent CO ₂ .	Per cent H ₂ .	Reaction.
24 hours				
48 hours				

		24 hours		48 hours		Remarks
		acid	non-acid	acid	non-acid	
Litmus lactose agar plates	1.0 c.c.					
	0.5 c.c.					
	0.1 c.c.					
						Average count
		3 days		7 days		
		liquef.	non-liquef.	liquef.	non-liquef.	
Gelatin plates	1 c.c.					
	0.5 c.c.					
	0.1 c.c.					
						Average count

Plates made from ferm't'n tube or agar shake.
 Organisms isolated.

BACTERIA ISOLATED FROM WATER SAMPLES

[illegible]

EXERCISE 3. TO DEMONSTRATE THE EFFICIENCY OF CHLORIDE OF LIME AS AN AGENT IN THE PURIFICATION OF DRINKING WATER

Apparatus. Small can of commercial chloride of lime (one can is sufficient for the class); 6 liter precipitating jar; sterile 1.5 liter flask; 20 sterile Petri dishes; sterile 1 c.c. pipettes; two sterile 100 c.c. volumetric pipettes; ten tubes of litmus lactose agar; ten tubes gelatin; two litmus lactose agar shakes; six dilution flasks, four 99 c.c. and two 90 c.c. (distilled water); one liter of sewage.

Method. 1. Using the large precipitating jar, prepare a 6% solution of chloride of lime (by weight), using the entire contents of a *newly opened* can. This stock solution will contain about 2% available chlorine calculated on the basis of 35% available chlorine in the commercial chloride of lime.

2. Mix well and allow to settle over night before proceeding with the experiment.

3. Obtain the sewage in the large sterile flask. Save a small quantity of the sewage in a sterile test tube for microscopic examination.

4. Make gelatin and litmus lactose agar plates of the fresh sample, plating 1 c.c. and 0.1 c.c. direct, and dilutions, 1-100, 1-10,000 and 1-1,000,000 (five plates in each medium).

5. Add 1 c.c. of the 6% solution of chloride of lime (2% available chlorine) to exactly 1,000 c.c. of the sewage in the sterile flask; shake *vigorously* for one minute and allow it to stand one hour. This amount is over ten times the amount necessary for sterilization, calculating on the basis of 16 lbs. of chloride of lime (containing 35% available chlorine) per million gallons of water. (See table on page 11, Hooker's Chloride of Lime in Sanitation.)

6. Plate in gelatin and litmus lactose agar, using 1 c.c.

and 0.1 c.c. direct, and dilutions of 1-100, 1-10,000 and 1-1,000,000 (five plates for each medium).

7. Make duplicate agar shakes also, with the raw river water and with the same after the chloride of lime has been added.

8. Incubate all plates and shakes at 37° C. Observe each at the end of twenty-four and forty-eight hours.

9. Count the agar plates at forty-eight hours and record the results. Count the gelatin plates before the colonies are obscured by liquefiers.

10. What types of organisms have been destroyed? What types remain? Is this according to the results of other investigators (see Hooker, p. 23). Would you feel safe in drinking this water?

11. Examine the sewage in a hanging drop and draw the types of organisms present. Note which types predominate, also note their comparative size and motility.

12. Make records of these to compare with the data on cultural determinations. Are all of these types found by the cultural methods?

13. Chloride of lime is used for purifying drinking water in the proportions of 5 to 25 pounds per million gallons of water. What effect does chloride of lime have on organic matter, discoloration, turbidity and swampy or other smells in raw water?

14. 35% available chlorine is necessary for efficient sterilization. The *available chlorine* is merely an index of the *efficiency* of the chloride of lime. *Chloride of lime* in its industrial applications of bleaching, deodorizing or disinfecting *does not act by its chlorine but by its oxygen. Its action is not chlorination but oxidation.* (Hooker, p. 7.)

15. What is the maximum limit for the amount of chloride of lime used in dosing drinking waters? What is the amount used for treating water on shipboard? Why?

What is the minimum length of contact allowed between

the stock solution of chloride of lime and the water to be purified? Why is a minimum time limit set?

What other means are used for the chemical sterilization of water? Are these efficient?

What is the Hazen theorem? Its explanation?

What other uses has chloride of lime in sanitation?

16. Give all data and observations in full. State any conclusions to be based on the above and point out any practical applications.

REFERENCES

- HOOKE: Chloride of Lime in Sanitation (1913), pp. 7, 12-34, 34-77.
WESBROOK, WHITTAKER and MOHLER: The Resistance of Certain Bacteria to Calcium Hypochlorite. Reprint from Jour. of American Public Health Association, Vol. I (1911).
WHITTAKER: Field Equipment for Laboratory Work on Water Supplies. Reprint from Journal of American Public Health Association, Vol. II (1912), pp. 948-954.

EXERCISE 4. TO DEMONSTRATE THE EFFICIENCY OF THE BERKEFELD FILTER CANDLE AS A MEANS OF WATER PURIFICATION

Apparatus. Berkefeld filter candle complete with cylinder; water-power vacuum pump; filter flask with rubber stopper into which the filter candle fits; one liter sterile flask; eight tubes of salt-free gelatin for water analysis; eight Petri dishes; sterile 1 c.c. and 10 c.c. pipettes; dilution flasks; distilled water

Method. 1. Set up the filtering apparatus, connect with the vacuum pump and wash the filter by running through it 500 c.c. of boiling, distilled water.

2. Place a cotton plug in the cylinder and in the side arm of the filter flask; sterilize the filtering apparatus as set up, in the autoclav.

3. Collect a liter of polluted river water from a point near the opening of a sewer.

4. Make dilution plates in gelatin immediately, using dilutions 1 : 10, 1 : 100, 1 : 10,000 and 1 : 1,000,000.

5. Filter the remainder of the sample through the Berkefeld filter candle and plate immediately from the filtrate, making gelatin plates, using 1 c.c. and 0.1 c.c. direct, dilutions of 1 : 100 and 1 : 10,000.

6. Incubate all plates at the same temperature. Examine the plates daily and record the counts.

7. Was the bacterial count reduced by the use of this filter candle? What type of organisms passed the filter? Is this filtered water fit for drinking purposes?

How does this filter compare with other types as to its action? What other types of filters are used for water purification? Sewage purification? Upon what does the value of each depend?

8. Give all data in full and draw any conclusions that are warranted. Point out any practical applications.

REFERENCES

MARSHALL: Microbiology, pp. 205-207.

DON and CHISHOLM: Modern Methods of Water Purification (1911), pp. 231-236.

SOIL MICROBIOLOGY

EXERCISE 1. TO TEST THE CATALYTIC POWER OF SOIL

Apparatus. 3% hydrogen peroxide; three 375 c.c. Erlenmeyer flasks; three one-hole stoppers to fit flasks; three pieces of bent glass tubing; three pieces rubber tubing; three 100 c.c. glass graduates.

Culture. Soil rich in humus.

Method. 1. Fit the Erlenmeyer flasks with one-hole rubber stoppers through which a short piece of bent glass tubing is inserted. Fit short pieces of rubber tubing (about 40 cm. in length) to the glass tubing.

2. Place 5 gms. of the soil in one of the flasks and mix it with 50 c.c. of water.

3. Arrange the 100 c.c. graduate in a water bath for collecting gas, by filling with water and inverting mouth down, under water. Clamp it in place.

4. Then add 20 c.c. of 3% hydrogen peroxide to the flask containing the soil, stopper the flask and, keeping the mixture moving, collect the oxygen in the graduated tube over water. Record the time for maximum oxygen liberation.

5. At the same time determine the part played by bacteria and enzymes, by repeating the catalase test on soil that has been heated in the autoclav at 15 lbs. pressure (120° C.).

6. Also determine the part played by humus by repeating this test with the same earth, having burned the humus by heating with the flame.

7. Record your results according to the diagram following:

	Fresh soil.	Autoclaved soil.	Burned soil.
Cubic centimeters of oxygen.			
Time for maximum oxygen liberation.			

8. What part does each factor play in the liberation of oxygen? Is this action in soil of any value? If so, what?

9. Compare your results with those of the catalase test in milk. By which substance is the most oxygen liberated? Is there a logical explanation for this?

10. Give all results in full, draw any conclusions that

are suggested and point out any possible practical applications.

REFERENCE

LÖHNIS: Laboratory Methods in Agricultural Bacteriology, p. 103.

EXERCISE 2. A COMPARATIVE STUDY OF THE NUMBER AND TYPES OF MICROORGANISMS IN SOIL: THE PLATE VS. THE MICROSCOPIC METHOD

A. THE PLATE METHOD

Apparatus. Sterile soil borer; sterile paper bag; sterile large spatula; sterile large piece of paper; sterile paper 15 cm. square; 500 c.c. wide-mouthed flask containing 200

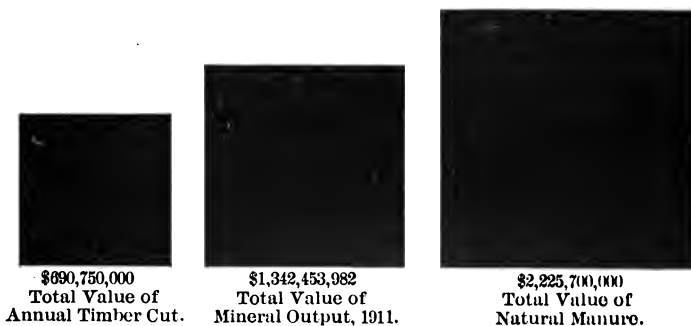


FIG. 54.—Showing the Comparatively Enormous Value of Organic Microbial Food which Should Enter the Soil. (Carver, Year-book of U. S. Dept. of Agr., 1914.)

c.c. sterile water; 90 and 99 c.c. dilution flasks (sterile water); ordinary agar; sterile Petri dishes; soil (different types), manure, etc.

Each student should use one type of soil and one type of manure for this experiment. Assignments will be made by the instructor. The results obtained by each student are to be compared with those of others.

Method. 1. Remove the coarser surface *débris* from the soil. Sink the soil borer to the depth of 30 cm., remove the borer and place the soil in the bag.

2. Take six borings so that your composite sample will be representative of the entire plot under consideration.

3. At the laboratory, carefully mix and pulverize the composite sample with the spatula on the large piece of paper.

4. Weigh out 20 gms. on sterile paper and transfer immediately to the flask containing 200 c.c. of sterile water. A large amount of soil is used to reduce the error as much as possible. The manure should be treated in a similar manner.

5. Shake thoroughly for one minute, allow the coarser particles to settle and transfer 10 c.c. (equivalent to 1 gm. of soil) of the supernatant liquid to 90 c.c. of sterile water. Each cubic centimeter of this dilution then contains 0.01 gm. soil.

6. Make and plate from the following dilutions: 1-10, 1-100, 1-1000, 1-10,000, 1-100,000, 1-1,000,000.

7. Incubate at room temperature for four to eight days.

8. Count and record the results as number of bacteria per gram of soil or manure, in tabular form.

9. Record the number of the various types of microorganisms. Note the numbers of chromogenic bacteria and the streptothrix forms.

10. Examine some of the manure in the hanging drop. What forms are seen? Make drawings. Are all of these forms found on the plates? Give reasons for what does occur. Add sterile water to the manure in a sterile deep culture dish or flask and examine every three or four days during the course of the experiment. Record your observations.

11. When the peat sample is obtained, at the same time

partially fill a small sterile flask with swamp or marsh water. Examine immediately in the hanging drop and draw the forms seen.

12. Place this swamp water in the sunlight (more or less direct) for two or three days and examine again in the hanging drop for any forms of life present.

13. Compare the flora of these two microscopical preparations. Suggest why each type of organism is present.

14. Compare all types of soil examined both quantitatively and qualitatively as to their microflora. Which soils are most alike in their flora? Suggest a reason why. Why do various soils vary in the number of bacteria found?

REFERENCES

MARSHALL: Microbiology, pp. 226-245.

CONN: Agricultural Bacteriology, pp. 34, 70, 120.

EYRE: Bacteriological Technic. Second Ed., pp. 470-478.

B. MICROSCOPICAL METHOD

Apparatus. Soil or manure of same type as used for plating; sterile water; sterile Chinese ink; platinum loop of known capacity; sterile watch glass; cover-glasses, *absolutely clean*; ocular micrometer; stage (object) micrometer.

Method. 1. To 1 gm. of soil or excrement in a test tube add 4 c.c. of sterile water and shake vigorously for five minutes.

2. Place 0.5 c.c. in a clean, sterile watch glass. Add 0.5 c.c. of Chinese ink.

3. Mix with a platinum loop of known capacity.

Note. To determine the capacity of the platinum loop, weigh two watch glasses. Into one put exactly 1 gm. (1 c.c.) of water. Transfer five loopfuls from the glass containing water to the empty watch glass.

Weigh each. Then determine the weight and also the volume of one loopful.

4. Transfer one loopful of the "ink manure" solution to a clean, sterile cover-glass and spread in an even film over the entire surface.

5. Let this dry in air and fix by passing three times through the flame. Mount at once in balsam.

6. Measure the surface area of the cover-glass. Also the diameter of one field of the oil immersion lens (using the stage micrometer) and from that the area of the field.

7. Count fifty fields and determine the average.

8. From the data which you now have, determine the number of organisms on the cover-glass, which is the number in one loopful.

9. Then from this calculate the number in 1 gm. of soil or excrement.

10. Also calculate the *weight* of bacteria in 1 gm. (See p. 88, Marshall's Microbiology.)

11. Compare the count thus obtained with the count obtained by the plate method. What is shown? How do you explain this result?

12. Compare the manure and soil counts. Draw conclusions and explain.

13. What other methods are used for obtaining numbers, etc., of organisms in soil and like materials?

14. State your data and observations in full. Draw any conclusions warranted and point out any practical applications.

REFERENCES

- LÖHNIS: Laboratory Methods in Agricultural Bacteriology, pp. 89-91.
LIPMAN and BROWN: Laboratory Guide in Soil Bacteriology, pp. 7-9.
MARSHALL: Microbiology, pp. 238-244.

EXERCISE 3. TO ILLUSTRATE THE EFFECT OF AERATION OF SOILS ON THE ACTIVITIES OF THE MICRO-ORGANISMS CONTAINED THEREIN

Apparatus. Coarse, medium, and fine sand; magnesium oxide; dilute HCl; N/10 NH₄OH; N/10 HCl; indicator; sterile 1 c.c. pipette; ten sterile 250 c.c. Erlenmeyer flasks; condenser; sterile 1% peptone solution.

Culture. *B. mycoides*, broth culture.

Method. 1. Prepare the sand for use by first heating it with dilute HCl; then wash it several times, first with tap water then with distilled water and dry at 110° C.

2. Place 50 gms. of coarse sand in each of three flasks; do the same with the medium and the fine sand.

3. Sterilize the flasks in the autoclav at 120° C. for thirty minutes.

4. Place 50 c.c. of the 1% peptone solution in the remaining flask.

5. Add sufficient peptone solution to the other flasks to make 10%, 20% and 30% moisture content according to the following table:

Soil.	Moisture.	After twenty days.
Coarse sand.....	10%	
	20%	
	30%	
Medium sand.....	10%	
	20%	
	30%	
Fine sand.....	10%	
	20%	
	30%	
1% peptone solution.....	99%	

6. Inoculate each flask with 1 c.c. from a broth culture of *B. mycoides* and shake to distribute the organisms evenly.

7. Make ammonia determinations after twenty days.

Note. Ammonia determinations from the above are made by the following procedure: Remove the cotton plugs, add 200 c.c. of water, 10 gms. MgO and a small piece of paraffin. Distill off the ammonia present, collecting it in N/10 HCl and titrating against N/10 NH_4OH , using methyl orange as indicator.

8. Record your results in the form given above.

9. How does aeration affect bacterial activity? Size of sand grains?

What interrelation have grain-size and moisture content of soil?

How do microorganisms obtain their food in soil that is not wet?

What influence does humus have on aeration? On bacterial activity?

10. Draw any conclusions warranted by your results and point out any practical applications.

REFERENCE

RAHN, O.: Tech. Bul. No. 6, Mich. Agr'l Expt. Sta.

EXERCISE 4. TO DEMONSTRATE THE CELLULOSE-DECOMPOSING POWER OF AEROBIC ORGANISMS FOUND IN THE SOIL

Apparatus. Two Petri dishes; four pieces of round filter paper to fit Petri dishes; 0.05% K_2HPO_4 ; MgNH_4PO_4 ; 0.05% NH_4NO_3 ; 1 c.c. pipette; soil rich in humus, or well-rotted manure.

Method. 1. Put a thin layer of MgNH_4PO_4 between two filter papers in a Petri dish.

2. Moisten this with the solution of K_2HPO_4 and inoculate with a few drops from a water solution of the soil or manure. Keep at 25° to 30° C.

3. In three to six days brown spots will occur and later holes will be formed by bacteria. Thin places in the filter

paper can be detected by holding the Petri dish towards the light.

4. With a sterile platinum needle, test the consistency of the paper in the spots which have been most probably attacked and compare with that of the undecomposed spots. Describe the results.

5. Add more of the K_2HPO_4 solution when necessary to keep the filter paper moist.

6. Start a second Petri dish in the same way but keep it moist with 0.05% NH_4NO_3 and 0.05% K_2HPO_4 . Here we find brown spots caused usually by fungi.

7. Macerate some of the brown spots from each Petri dish with water and make a Chinese ink preparation.

8. What types of organisms are seen in each preparation? Make drawings.

9. What organisms are especially active in the anaerobic decomposition of cellulose? Which type, aerobic or anaerobic, is responsible for the greater amount of cellulose decomposition in nature? When may the other types take precedence?

What steps would you take to isolate the organisms which are growing on your plates?

Are the chemicals used above present in the soil? In what form? In what forms does cellulose exist in cultivated soils?

Are cellulose-decomposing bacteria ubiquitous? Are they always found where cellulose in some form is deposited? Are cellulose decomposing bacteria limited to soil?

10. Data and results are to be given in full, also draw any conclusions warranted and point out any possible practical applications.

REFERENCES

- LÖHNIS: Laboratory Methods in Agricultural Bacteriology, pp. 104-105.
LIPMAN and BROWN: Laboratory Guide in Soil Bacteriology, pp. 62, 63, 65.

MARSHALL: Microbiology, pp. 246-249.

McBETH and SCALES: The destruction of cellulose by bacteria and filamentous fungi. Bul. 266, B. P. I., U. S. Dept. Agr. (1913).

EXERCISE 5. TO ILLUSTRATE THE ANAEROBIC DECOMPOSITION OF CELLULOSE BY SOIL AND FECAL ORGANISMS

Apparatus. Six (large) tubes of Omelianski's synthetic medium for anaerobic cellulose fermentation; tall Novy jar; vacuum pump.

Culture. Fresh and decayed manure.

Method. 1. Inoculate one tube with small amounts of fresh horse or cow manure, a second with partially decayed manure.

2. Place some cotton in the bottom of the Novy jar, insert the inoculated tubes in it, replace the stopper and exhaust the air by means of the vacuum pump. (Pyrogalllic acid and sodium hydroxide may be substituted.)

3. Incubate the tubes in the Novy jar at 34° to 35° C. for four to six weeks.

4. From time to time note any changes occurring in the filter paper.

5. After the latter has been wholly or partially digested, make transfers to new tubes of the medium and incubate anaerobically as before.

6. Repeat this procedure from the cultures made just previously. (The jar is evacuated each time after observations are made.)

7. Examine the organisms causing the disintegration of the filter paper both in the hanging drop and with some ordinary stain (not the ink preparation). Make permanent stained preparations.

8. Starch, cotton, straw, etc., digestion may be compared if these substances are substituted for filter paper in Omelianski's medium.

Not taking soil into consideration, where do anaerobic

cellulose-decomposing organisms probably play a most important part? How is this determined?

9. What types of anaerobic cellulose-decomposing bacteria are favored by this synthetic medium? These bacteria have only in exceptional cases been grown on solid media. How can these types be separated?

10. Data and observations should be given in full. Draw any conclusions warranted and indicate any practical applications that may be made.

REFERENCES

- LÖHNIS: Laboratory Methods in Agricultural Bacteriology, p. 93.
LIPMAN and BROWN: Laboratory Guide in Soil Bacteriology, p. 64.
MARSHALL: Microbiology, pp. 246-248.
McBETH and SCALES: l.c., Exercise IV.

EXERCISE 6. TO ILLUSTRATE NITRIFICATION IN SOLUTION

Apparatus. Nine 50 c.c. Erlenmeyer flasks; 75 c.c. each of Solutions I, II and III for nitrification (see appendix); Nessler's solution; *α*-naphthylamin; sulphanilic acid; 2% diphenylamin in sulphuric acid; aqueous alcoholic stains.

Cultures. Rich soil from cultivated land; manure from surface layers of manure heap.

Method. 1. Place 25 c.c. of each solution in a 50 c.c. Erlenmeyer flask and sterilize in the autoclav.

2. Inoculate as follows:

	Solution I.	Solution II.	Solution III.
0.5 gm. manure.....			
0.5 gm. soil.....			
Nothing—control.....			

and keep at 25° to 30° C., to hasten the action of the micro-organisms.

Owing to the presence of some carbon-monoxide in the air of the laboratory from the burning gas, the carbon-monoxide-assimilating *B. oligocarboophilus* often appears as a dry white skin on the surface of the solution in these flasks.

Note. Solution I is adapted for relatively increasing the nitrite bacteria, Solution II the nitrate producers and Solution III the simultaneous growth of both organisms as in nature.

3. After eight to fourteen days, test all solutions and control flasks every second or third day by transferring 0.1 c.c. with a sterile pipette to a white glazed surface (e.g., plate) using

- (a) Nessler's solution, for ammonia;
- (b) nitrite test solutions, for nitrites;
- (c) nitrate test solution, for nitrates.

Tabulate your results. Discuss and explain the decomposition which is taking place in each inoculated flask, giving the successive steps in the disintegration of the crude nitrogenous organic matter.

4. Examine a loopful of each solution in the hanging drop each time chemical tests are made. Morphologically what types predominate in each solution? In the sample of soil? Of manure? Are any of these spore-formers? If so, which type?

5. Make permanent stained preparations from each flask.

6. Nitrifying bacteria do not grow on the ordinary solid media. Why? Many different methods have been tried for the isolation of nitrifying organisms but the obtaining of pure cultures is still a most difficult bacteriological task.

(See appendix for media used for the isolation of these organisms.)

What methods are employed for their isolation besides the use of solid synthetic media? What is the principle of each method?

What are the different types of nitrifying organisms?

Their respective functions? What interrelationships exist between these organisms?

Where are nitrifying organisms found in nature? What is their significance?

What conditions in soil are necessary for their proliferation? What methods does the agriculturist use which serves to conserve these organisms?

REFERENCES

- LÖHNIS: Laboratory Methods in Agricultural Bacteriology, pp. 96, 97, 106, 109, 110.
LIPMAN and BROWN: Laboratory Guide in Soil Bacteriology, pp. 25-34.
MARSHALL: Microbiology, pp. 259-263.

EXERCISE 7. TO ILLUSTRATE DENITRIFICATION IN SOLUTION

Apparatus. Eight tubes of sterile Giltay's solution; eight tubes of nitrate broth; eight to ten tubes of Giltay's agar; eight to ten tubes of nitrate agar; eight fermentation tubes of nitrate bouillon, four with and four without sugar (use 1% dextrose); peptone solution in tubes; sterile 1% solution of KNO_3 ; nitrate test solution; Nessler's solution, test for NH_3 ; α -naphthylamin and sulphanilic acid (nitrite test solutions); sterile 1 c.c. pipettes; sterile Petri dishes; sterile dilution flasks.

Cultures. Soil (record type used); straw from manure pile; horse manure.

Method. 1. Inoculate test tubes of each solution in series as follows:

1 and 2—

3 and 4—0.1 gm. soil.

5 and 6—0.1 gm. straw;

7 and 8—0.1 gm. horse manure.

2. Incubate these at 37° C. for forty-eight hours.

3. Note any changes occurring. Determine whether

any nitrites or ammonia have developed. To what are the gas bubbles due?

The crystals deposited in Giltay's solution are magnesium phosphate.

4. Using sterile pipettes, test 1 c.c. portions of each after forty-eight hours, seven days, etc., for nitrates with phenolsulphonic acid.

5. From two tubes showing abundant gas formation, make nitrate agar plates, using a wide range of dilutions that one or two plates may show well-isolated colonies. Incubate at 37° C.

6. From various colonies appearing on the plates, make stab cultures in nitrate agar. Incubate these at 37° C.

Save one showing the most abundant gas formation under these conditions, for further study.

7. Inoculate a nitrate bouillon fermentation tube with the pure culture just isolated, also add some of the crude material to a fermentation tube.

8. Duplicate with a nitrate bouillon fermentation tube containing sugar.

9. Determine the amount and nature of the gas formed in each tube and compare results. (Determine by elimination; test for CO₂ and H₂.)

What influence does dextrose have upon the rate and amount of gas formation?

10. Inoculate in duplicate, tubes of peptone solution with the pure culture. Record the growth and gas formation, if any, qualitatively.

11. To an old culture (not necessarily pure) in which the nitrates have disappeared, add 1 c.c. of a sterile 1% solution of KNO₃. Does gas formation re-occur?

12. Continue to add a small amount of KNO₃ as rapidly as the culture ceases to give a reaction for nitrates (an indication that the latter have been used up.) Note how much KNO₃ your culture can reduce.

13. Theoretically, what would be the difference in

action of denitrifying organisms in soil and in solution? Why?

How do denitrification and nitrate reduction differ?

How may colonies of nitrate-reducing bacteria be detected?

14. Give all results in full and draw conclusions. Suggest any possible practical applications of the above.

REFERENCES

- LIPMAN and BROWN: Laboratory Guide in Soil Bacteriology, pp. 35-40.
 MARSHALL: Microbiology, pp. 263-267.
 LÖHNIS: Laboratory Methods in Agricultural Bacteriology, pp. 98-99.

EXERCISE 8. TO ILLUSTRATE THE NON-SYMBIOTIC FIXATION OF NITROGEN BY SOIL ORGANISMS, AND ISOLATION OF AZOTOBACTER THROUGH ITS MINERAL FOOD REQUIREMENTS

Apparatus: Mannit solution for nitrogen-fixing organisms; mannit agar; eight sterile 100 c.c. Erlenmeyer flasks; concave slides; cover-glasses; concentrated H_2SO_4 ; filter paper.

Culture. From clay loam, sandy loam, manure.

Method. **1.** Place 50 c.c. of the mannit solution in each flask and sterilize (in autoclav).

2. Inoculate in series as follows:

Flasks 1 and 2—

Flasks 3 and 4—0.1 gm. clay loam.

Flasks 5 and 6—0.1 gm. sandy loam.

Flasks 7 and 8—0.1 gm. manure.

3. Incubate at room temperature and note the changes taking place. A wrinkled skin, white at first, brownish later, is gradually formed. This is composed of the aerobic *Azotobacter*.

4. From time to time examine the cultures in the hanging drop and note the type of organisms predominating. Single, small, thin bacilli are visible between the large

cells of *Azotobacter*. The former are almost always a type resembling the nodule bacteria, *B. radiobacter*, and which can also fix nitrogen to a slight extent. Besides these, especially when *Azotobacter* is less in evidence, many other sporing and non-sporing bacteria participate in the process. *Azotobacter*, however, is the most vigorous free nitrogen-



FIG. 55.—*Azotobacter*. x1000; Smear from Six-day Old Culture on Ashby's Agar at 25° C. Showing organisms and capsules in various stages of development. (Dan H. Jones.)

fixing organism yet discovered. (See reference, E. B. Fred, Exercise 9, Soil Microbiology.)

5. What characteristic odor is produced in these cultures? Add a drop of concentrated H_2SO_4 to a small portion of the culture liquid. This intensifies the odor.

6. When a brownish surface film develops, make plates from this culture, using relatively high dilutions.

7. After a rather long period of incubation (six to seven days) examine the organisms in the various colonies and isolate *Azotobacter chroococcum* if possible upon a mannit agar slant. On account of the slimy property of its cell wall, its separation from *B. radiobacter* is often very difficult. The quickest way is to reinoculate first into the mannit solution.

8. Save several of the plates having well-isolated colonies and note any changes which may occur.

9. If any brown colonies develop, examine them in stained preparations. Measure the bacteria stained.

10. Are these pure cultures? If not, plate from several such colonies in mannit agar to isolate the different organisms present.

11. Is *B. radiobacter* present? What part does it play in the fixation of nitrogen?

12. Make several agar slant pure cultures of *B. radiobacter*.

13. Study the morphology and the cultural characteristics of this organism.

14. Inoculate a small flask of the mannit solution with a pure culture of the newly isolated organism.

15. How does this organism compare, morphologically, culturally, etc., with *Ps. radicola*? With the *Azotobacter*?

What part in the nitrogen cycle does the *Azotobacter* play?

What practices of the farmer favor the development of the *Azotobacter*? In what way? What soil conditions are favorable to the *Azotobacter* species? Are these conditions favorable to other bacteria? To plants?

Diseased spots in soil are said to be caused by an excessive nitrogen fixation and nitrification, e.g., the niter spots in Colorado soils.

16. State your results in full and draw conclusions. Point out the practical applications of the above.

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EXERCISE 9. A STUDY OF THE SYMBIOTIC NITROGEN-FIXING ORGANISMS OF LEGUMES, *PS. RADICICOLA***A. ISOLATION OF *PS. RADICICOLA* FROM NODULES OF *LEGUMINOSÆ***

Apparatus. Spade or trowel; sterile Petri dishes; tubes of nitrogen-free ash agar; tumbler for mercuric chloride solution; small piece of filter paper; small pair of forceps; scalpel or chisel-edged platinum needle; platinum loop; clean slides; mercuric chloride, 1-500; alcohol, 95%; tubes of sterile water; aqueous-alcoholic gentian violet or fuchsin; eosin; Lugol's iodine solution; saturated alcoholic solution of gentian violet.

Culture. From nodules of roots of leguminous plants.

Method. 1. Using a spade or trowel, obtain the roots of some legumes which show nodule formation in abundance. If the soil is firm, as with clay, do not forcibly pull up the legume to obtain the roots as this procedure strips off the nodules which develop almost without exception on the young rootlets.

Note. Heavily inoculated legumes may be stored for winter use in a cool, dry, dark place. Living organisms have been found after more than two years in some of the larger nodules.

2. Thoroughly wash the roots under the tap and place the plant in clean, cool water.

3. Keep parts of the plant for identification if the species is unknown. Not all plants belonging to the family *Leguminosæ* are attacked by symbiotic nitrogen-fixing bacteria, only those belonging to the sub-family, *Papilionaceæ*.

4. Compare the size, numbers, and location of the nodules on the roots of the different legumes.

5. Remove a good-sized nodule from the roots, wash in clean water and immerse for three minutes in mercuric chloride solution, 1 : 500.

6. Remove the nodule with flamed forceps and take up the excess of the solution between folds of sterile filter paper, then dip into alcohol, the last traces of alcohol being removed by passing the nodule quickly through the flame.

7. Place the nodule on a flamed and cooled slide.

8. Holding the nodule in flamed and cooled forceps, cut into it, and break it open by means of a sterile scalpel or a chisel-edged platinum needle.

9. Thrust a sterile platinum needle into the nodule in the middle of the newly exposed surface and gently rotate the needle so that some of the crushed tissue adheres to it.

10. Touch the needle in a drop of sterile water in a sterile Petri dish.

11. Transfer a loopful of this suspension to a second drop of sterile water in a second Petri dish and a loopful from this to a third drop in a third Petri dish.

12. Pour the plates, using tubes of nitrogen-free ash agar and incubate at room temperature for two or three days.

13. Make a smear on a clean slide from the freshly cut surface of the nodule, stain and examine microscopically. What is the morphology of *Ps. radicola* found in the nodule? What are bacteroids?

14. Make a smear directly from this same nodule on a clean slide, fix and stain with eosin followed by Lugol's iodine solution. The iodine demonstrates the starch which is usually present in nodules. Is starch present?

15. After a few days of incubation the colonies of *Ps. radicola* will be noted on the plates as round, grayish-white, translucent, slime-like drops, finely granulated and often with compact white centers.

16. Examine these colonies in the hanging drop. They contain the normal, short, rod forms which during the first days are very motile.

17. Isolate several pure cultures of *Ps. radicola* on

slanted nitrogen-free agar and note characteristic growth. Why is nitrogen-free agar used for the cultivation of *Ps. radicum*?

18. Make permanent stains of pure culture and compare with organisms on stained smear from nodule as to size, shape, etc. Are involution forms present in either preparation?

Do all species produce organisms of the same general morphology in the respective nodules? In pure culture?

19. Make a flagella stain from the pure culture as follows:

a. Take a loopful of the mucilaginous growth from a colony or an agar culture and spread it on a clean slide, lashing it out in slender tongues.

b. Let the film dry in air without killing or fixing.

c. Flood the film a moment with saturated alcoholic solution of gentian violet.

d. Wash under the tap, dry and examine with the oil immersion lens.

20. The mucilage in which the cells lie will be found to be deeply and evenly stained and the bacteria stained scarcely at all, so that the preparation presents somewhat the appearance of a photographic negative.

The single polar flagellum may be demonstrated by this stain, since it, like the protoplasm of the cells, refuses the stain, and so it appears as a clear, uncolored streak in the surrounding deeply stained mucilage. The flagella are best seen at the margins and in thin places, inasmuch as the mucilage in the denser areas masks the slender flagella.

21. Sometimes the roots of leguminous plants show, instead of the normal nodules, lesions of crown-gall caused by *Bact. tumefaciens* which somewhat resemble *Ps. radicum*.

22. For a rapid diagnosis, plate in the synthetic Congo red medium which differentiates these two organisms;

Ps. radiculicola forms white colonies, while *Bact. tumefaciens* absorbs the Congo red and therefore produces red or reddish colonies.

B. TEST OF THE PHYSIOLOGICAL EFFICIENCY OF *PS. RADICICOLA* AND OBSERVATION OF NOD- ULE FORMATION

To observe nodule formation and nitrogen fixation, it is necessary to have seeds germinating free from bacteria.

Apparatus. 500 c.c. of nitrogen-free agar; six sterile large test tubes with foot; sterile ordinary test tubes (the agar should be distributed in all the test tubes to a depth of about 5 cm.); sterile Petri dishes; clean slides; mercuric chloride, 1 : 500; flask of sterile distilled water; sterile pipette; seeds of some leguminous plant. (The smaller seeds are better for this experiment.)

Culture. *Ps. radiculicola* (specific strain).

Method. 1. Obtain sound, mature pods of some legume as pea, bean, vetch, etc.

For testing the physiological efficiency of the pure culture of *Ps. radiculicola* just previously isolated, use seeds from the same legume as that from which this particular culture was isolated.

2. Soak the pods for five minutes in mercuric chloride 1 : 500 and remove the excess of solution with sterile cheesecloth.

3. Tear open the pods with flamed forceps, place the seeds between folds of sterile cotton, and put the cotton in a dry, warm place until the seeds are dry.

4. Select the best of these seeds and store them in dry sterile test tubes until they are to be used.

5. Whether seeds are procured as described above, or otherwise, proceed as follows:

(a) Soak the leguminous seed in 1 : 500 mercuric chloride solution for five minutes.

(b) Wash off the disinfectant with sterile distilled water, handling the seeds with sterile forceps.

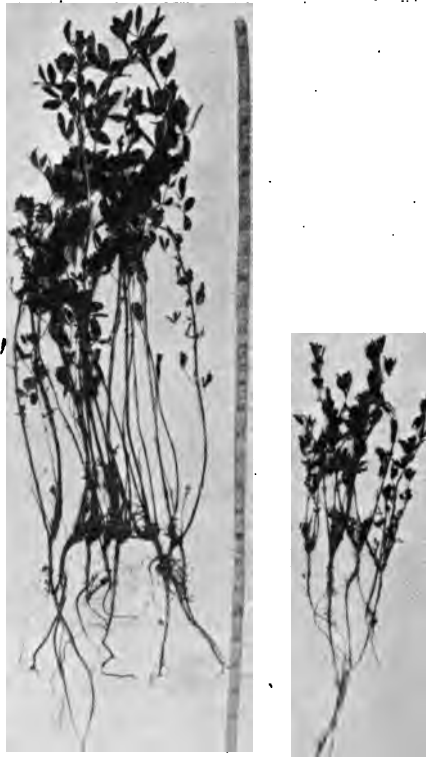


FIG. 56.—Alfalfa Plants from Inoculated and Uninoculated Seed.
(Orig. Northrup.)

6. Seeds prepared as above should then be treated according to the following procedure:

Using the sterile forceps, transfer several of these ster-

ilized seeds to each of the large test tubes. Or, place the seeds between layers of moist sterile filter paper in a Petri dish until they have germinated and then transfer the seeds to the large test tubes. When using the larger seeds use only three to six per tube, and six to ten *only* of the smaller seeds as alfalfa, clover, etc.

7. Put the tubes containing the ungerminated seed in a warm place (30° to 35° C.) until the seeds germinate. Keep the germinated seed in a well-lighted room for a few days.

8. Examine the tubes and reject all that are contaminated with molds or bacteria.

9. After a few days, inoculate four of these tubes containing growing leguminous plants with a pure culture of *Ps. radiculicola*, by dropping upon the seeds and surface of the agar a heavy suspension of the bacteria in sterile water, by means of a sterile pipette.

10. Keep two tubes uninoculated as controls.

Note. To imitate infection under more natural conditions, just before the seeds are placed upon the agar, the agar may be melted, cooled to 40° to 45° C. and inoculated with a loopful of *Ps. radiculicola* culture, mixing the organisms well through the agar with the needle. After the agar has solidified the sterile seeds may be then placed on the surface of the agar as before.

11. Label the test tubes and place in some location where they will be sufficiently protected from the sun, heat, or cold, etc. *This is very important.* A piece of cheese-cloth thrown over the tubes will protect them from the sun.

12. In about a month examine all test tubes and look for nodules.

13. Record the presence, number, size, and shape of nodules, place of formation, etc. Show nodule-bearing seedlings to the instructor.

14. Isolate *Ps. radiculicola* from one of these nodules.

This completes the cycle.

If all of these operations are successful Koch's postulates have been fulfilled. (See reference, W. J. MacNeal, Exercise 1, Animal Diseases and Immunity.)

15. What do you conclude as to the physiological efficiency of the culture of *Ps. radiculicola* used?

16. What several factors might be responsible for a failure of infection? Explain.

Why are inoculated seeds kept from direct sunlight?

What may be the advantage of seed inoculation? How



FIG. 57.—*Ps. radiculicola*, Polar flagella, x1500. Twenty-five day old culture from sweet pea. (B. Barlow).

do different methods of seed inoculation compare as to advantages and disadvantages?

17. Give your results in detail and draw conclusions. Point out any possible practical applications.

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EXERCISE 10. TO DEMONSTRATE THE CHANGE OF INSOLUBLE PHOSPHATES TO A SOLUBLE FORM THROUGH THE AGENCY OF MICROORGANISMS

Apparatus. Dextrose; di- or tri-calcium phosphate; tubes of soil-extract agar containing 2% dextrose; four 100 c.c. Erlenmeyer flasks.

Culture. Soil.

Method. 1. Place 0.1 gm. of di- or tri-calcium phosphate, and 60 c.c. of a 2% solution of dextrose in tap water in each flask. Sterilize.

2. To two flasks add 0.1 gm. soil each, leaving two for controls.

3. Incubate at 37° C., and after the fermentation has continued for some days, make plates from the inoculated flasks as follows:

4. Sterilize about 0.1 gram of di- or tri-calcium phosphate in each of three test tubes.

5. Place the contents of each tube in a sterile Petri dish; make loop-dilution plates from flasks in soil extract agar containing 2% dextrose, being careful to mix the phosphate well with the agar in the dish by carefully tilting.

6. Incubate at 37° C.

7. Note frequently the appearance of the plates. The colonies of acid-producing bacteria developing at this temperature dissolve the phosphate and thus become surrounded by a clear area similar to that produced by lactic acid-producing bacteria on dextrose calcium carbonate agar.

8. Examine the colonies in a hanging drop for morphology, motility, etc.

9. How is the action noted in 7 made use of practically?

In what compounds is phosphorus found in soil? Are these available as plant food? What are the functions of bacteria in this connection?

What relation has phosphorus to decay and nitrogen fixation?

10. Give results and any conclusions in detail. Point out any possible practical applications.

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DAIRY MICROBIOLOGY

EXERCISE 1. A COMPARATIVE STUDY OF THE NUMBER AND TYPES OF MICROORGANISMS AND OTHER CELLS IN MILK

A. PLATING METHOD

Apparatus. Sterile Petri dishes; sterile 1 c.c. and 10 c.c. pipettes; tubes of sterile litmus lactose agar; 90 c.c. and 99 c.c. dilution flasks; tubes of sterile litmus milk.

Culture. Fresh milk from any source desired.

Method. 1. Shake the milk vigorously one hundred times to obtain a homogeneous sample, and plate in dilutions, 1-100, 1-10,000 and 1-1,000,000 in litmus lactose agar.

2. Incubate the plates inverted at room temperature for five days.

3. Count at the end of this time, estimate the average number of bacteria per cubic centimeter and approximate the numbers of the different types of colonies.

4. Are acid colonies present? Chromogenic colonies? *B. subtilis* or *B. mycoides*?

What do the types signify?

5. Isolate the different types in litmus milk and note their action. To which group of microorganisms does each type belong? (See Marshall's Microbiology, pp. 306-313.) Suggest from what source each type may come.

B. MICROSCOPIC METHOD

Apparatus. Special capillary pipettes graduated to deliver exactly 0.01 c.c.; clean glass slides; three staining jars; xylol; alcohol, 95%; Loeffler's alkaline methylen blue; stage micrometer; eyepiece micrometer for counting objects in microscopic field; stiff straight needle.

Culture: Fresh milk—same as used in A.

Method. 1. Draw with ink a figure the size and shape of an ordinary microscopic slide and on either side and equidistant from the center draw a square whose area is one square centimeter, making the homologous sides of all figures parallel.

2. Place a clean glass slide on the figure.

3. With the capillary pipette, drop over the center of one of the smaller figures exactly 0.01 c.c. of milk directly from the well-shaped sample and with a stiff straight needle spread this drop of milk exactly over the area (one square centimeter) covered by this figure.

4. Make a duplicate smear, placing the drop of milk containing 0.01 c.c. on the slide over the remaining small square.

5. These smears may be dried by the use of gentle

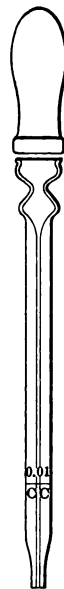


FIG. 58.—Capillary Pipette used in the Microscopic Method for Counting Bacteria in Milk. Note the straight narrow bore and the square tip.

heat (e.g., level wooden surface over a steam radiator). Do not allow the smears to become too hot, as this causes them to check, making satisfactory staining impossible.

6. As soon as dry, place the slides in a staining jar containing xylol for a short time to remove the fat.

7. Remove the slide from the xylol, absorb the surplus xylol about the edges with filter paper and allow it to dry.

8. Fix the film to the slide by immersing in 95% alcohol.

9. Stain immediately by flooding the smears with Loeffler's methylen blue for two or three minutes.

10. Decolorize to a light blue in 95% alcohol.

11. In counting, use the oil immersion objective. Place the draw tube at some convenient mark so that an even number of fields of the microscope covers one square centimeter.

To do this, determine the radius of the microscopic field in millimeters with the stage micrometer and calculate its area by the formula πR^2 . ($\pi=3.1416$.)

Then if x =the area of the smear in square millimeters and if 0.01 c.c. of milk is used,

$$\frac{x}{\pi R^2} \times 100 = y.$$

y =the factor necessary to transform the number of bacteria found in one field of the microscope into terms of bacteria per cubic centimeter.

To simplify the calculation, place the draw tube so that y consists of as many ciphers as possible. Convenient factors will be obtained if the length of R be 0.101 mm. or 0.08 mm.

Let z thousand equal the number of fields of the microscope in one square centimeter. Since 0.01 c.c. of milk was taken then each bacterium seen in one field represents $100 \times z$ thousand or z hundred thousand bacteria per cubic centimeter.

12. For careful quantitative work it is necessary to count one hundred fields for each sample, i.e., fifty fields per square. If n = the number of fields counted and m = the total number of bacteria found, the number of bacteria per cubic centimeter is calculated by the following formula:

$$\frac{z \text{ hundred thousand}}{n} \times m = \text{number of bacteria per cubic centimeter of milk.}$$

In comparatively fresh milk where the bacteria are few, count the whole microscopic field.

An eye-piece micrometer having a large square ruled into smaller squares is recommended where large numbers of bacteria are present. The area of the large square is different from that of the whole microscope field and consequently the factor necessary for computation is different. This factor can be determined by modification of the formula given in 11.

13. Draw a typical smear from different samples of milk. Indicate the kinds of cells and the number found, also the quality of the milk.

Quality of milk.	Bacteria per field.	No. per c.c.	Tissue cells.	Cell count.
Good	None	2	800,000 per c.c.
Fair	5	2,000,000	1	400,000 per c.c.
Souring normally	200	80,000,000	1	400,000 per c.c.
Poor	250	100,000,000	7	2,800,000 per c.c.

14. What types of bacteria are found microscopically? How do these compare with those found on plates as to types and numbers?

What are the advantages and disadvantages of the plating method? Of the microscopic method? For what type of work is each best adapted? What other microscopic

methods have been employed as a rapid means of setting bacteriological milk standards?

Of what value are bacteriological milk standards and analyses?

15. Give your results in detail and point out any practical applications.

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EXERCISE 2. THE DETERMINATION OF THE BACTERIAL CONTENT OF MILK IN THE UDDER

Apparatus. Several large sterile test tubes; four sterile Petri dishes; 99 c.c. dilution flasks; sterile 1 c.c. pipettes; tubes of sterile litmus milk; four tubes litmus lactose agar.

Method. 1. Wash off the end of the teat very carefully with a solution of mercuric chloride, 1 : 1000; allow it to dry till the surplus solution has disappeared and only sufficient moisture remains to make the cells and any dirt adherent.

2. Secure a sterile cotton-plugged test tube, remove the cotton plug with the little finger and, while holding the mouth of the tube as near the end of the sterilized teat as possible and inclining the tube toward the horizontal position as far as feasible, milk the tube half-full.

BACTERIAL CONTENT OF MILK IN THE UDDER 269

By this method obtain from the same teat:

- a. One sample of the fore milk,
- b. One sample of the middle milk;
- c. One sample of the strippings;

Note. For investigational purposes it may be better to employ a sterile milking tube adjusted to a sterile flask. This may easily be prepared. This method, however, is not recommended for student work.

3. Secure one sample from the pail, gathered from the same cow at the same milking.

4. Plate each sample on litmus lactose agar, using the following dilutions and amounts:

1 c.c. of 2a diluted 1 : 100 for the plate.

1 c.c. of 2b diluted 1 : 10 for the plate.

1 c.c. of 2c diluted 1 : 10 for the plate.

1 c.c. of 3 diluted 1 : 100 for the plate.

5. Place the plates at a temperature of 21° C. for seven days.

6. Count the number of colonies in each plate and record the average number in 1 c.c. of milk in each case. Explain any variation in counts.

7. Compare the colonies of plates 2a, 2b, and 2c with

3. What types predominate?

8. Estimate, so far as possible, the number of colonies of each type, and compare the relative numbers of each species in the different plates.

9. Isolate the species in milk tubes to study their action upon milk. To what group of microorganisms found in milk does each of the species isolated belong? How do you account for the presence of these particular species?

10. In which sample would you expect to find the greatest number of microorganisms? Why?

Why should all samples be taken from the same quarter of the udder?

How do bacteria ordinarily gain entrance to the udder?

By what means may bacteria cause infection of the udder?

What is the significance of bacteria in the udder? As to numbers and types?

11. Give your results in full and point out any conclusions and any practical applications possible.

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EXERCISE 3. TO ILLUSTRATE EXTRANEOUS CONTAMINATION

Apparatus. Forceps; scalpel or spatula; seventeen sterile Petri dishes; three 10 c.c. dilution flasks (for A and B); fifteen sterile 1 c.c. pipettes; fifteen tubes of sterile litmus lactose agar; soap; ordinary towel; two 1 qt. sterile basins; one milk pail; two 1 liter flasks each containing 500 c.c. sterile salt solution; one deep Petri dish; sterile glass rod.

A. SCALES FROM COW'S SKIN (DEAD EPITHELIAL CELLS)

Method. 1. With a sterile spatula scrape from the skin of a cow's udder some scales, such as usually fall into the milk, into a sterile Petri dish.

2. Transfer by means of the sterile spatula some of these to a 75 c.c. Erlenmeyer flask containing 10 c.c. of sterile physiological salt solution.

3. Shake thoroughly, then plate 1 c.c. of this suspension in litmus lactose agar.

B. HAIRS FROM COW

Method. 1. Select two hairs from the back of the cow where the usual or natural cleanliness exists and two from the hip stained with manure. By means of sterile forceps place them in sterile Petri dishes.

2. One of each kind, that is, one from the back and one from the hip, place in 10 c.c. of a sterile salt solution, as under A.

3. Shake thoroughly, then plate 1 c.c. of this suspension in litmus lactose agar.

4. Embed each of the two remaining hairs in the litmus lactose agar after pouring the liquefied agar into a sterile Petri dish. These hairs should be placed in the agar just before solidifying by means of sterile forceps.



FIG. 59.—*Bact. bulgaricum* colony, x75. (Orig. Nor-thrup.)

C. OTHER SUBSTANCES

Method. Study straw, hay, dung, etc., in a similar manner. Instead of using dilution flasks containing 10 c.c. it will be more desirable to use 100 c.c.

In the case of dung, a particle smaller than the head of a pin should be added to 100 c.c. for suspension, and in case of straw and hay very small segments unless they are very clean.

Note. A sufficient number of such substances should be studied to familiarize the student with the amount of contamination which may take place from these sources.

D. HANDS

Method. 1. Wash the hands in the ordinary manner, rinse them thoroughly, then wipe with an ordinary towel.

2. After this has been done, put 500 c.c. of sterile water in a sterile dish, and rub the hands thoroughly with this water.

3. Plate 1 c.c. of this water in litmus lactose agar.

4. Again rub the hands, before they have been washed and after working for some time, in 500 c.c. of sterile water placed in a sterile dish.

5. Plate 1 c.c. of this water in litmus lactose agar.

6. Compare the numbers (using 1 c.c. as the unit) and kinds of bacteria in the two plates.

E. PAILS

Method. 1. Add to a milk pail washed in the usual manner, 500 c.c. of sterile salt solution, and plate in litmus lactose agar, 1 c.c. of this suspension after it has been moved over the inner surface of the pail.

2. Repeat by using a milk pail heated in steam for ten minutes, or cleansed with boiling water.

3. This same process may be repeated using milk bottles, milk cans, etc.

F. AIR

Method. 1. Determine qualitatively the microflora of the air of the stable before feeding or bedding or before any disturbing, and after feeding or bedding or after any disturbing, by the following methods:

2. Pour the liquefied litmus lactose agar into several Petri dishes, and expose the poured plates for different lengths of time.

3. Expose 10 c.c. of sterile 0.6% salt solution in a deep Petri dish 5 c.c. deep and 9 c.c. in diameter. Try to disin-

tegrate the dust particles by stirring with a sterile glass rod and agitating. Plate 1 c.c. in litmus lactose agar.

4. Quantitative studies of barn air under various conditions may be made according to Exercise 1, Air Microbiology.

5. What advantage has litmus lactose agar over ordinary agar in this exercise?

What types of organisms are met most frequently under A, B, C, D, E and F? How may this occurrence be accounted for?

Which sources furnish the greatest number of organisms? From which sources are the greatest number of microorganisms most likely to enter milk? The most undesirable types? Explain in each case.

What sources of milk contamination have not been discussed under this exercise? Of what importance is each? What is the simplest method in each case of preventing contamination from the various sources mentioned above?

6. Give your results in full and draw any conclusions and make any practical applications possible.

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EXERCISE 4. TO INVESTIGATE THE AMOUNT AND KIND OF DIRT IN MILK AND ITS RELATION TO THE MICROBIAL CONTENT OF THE MILK

Apparatus. Six sterile 1 c.c. pipettes; 99 c.c. dilution flasks; six tubes sterile litmus lactose agar; six sterile Petri dishes; sedimentation tubes, 10 c.c. capacity; balance; centrifuge; clean slides; methylen blue, aqueous-alcoholic; physiological salt solution; pneumatic or other type of

sediment tester; cotton disks for sediment tester; clean empty milk bottle; one pint bottled milk from each of several miscellaneous sources.

Note. The same sample of milk must be used for A, B and C. Proceed with tests in the order given.

A. DETERMINATION OF MICROBIAL CONTENT OF MILK

Method. 1. Shake the sample in the bottle vigorously.

2. Plate the dilutions 1 : 100, 1 : 10,000 and 1 : 1,000,000 in litmus lactose agar.

3. Place the plates at 25° C., and proceed with the microscopic sediment test.

4. Count the plates at the end of five days and estimate the number of bacteria per cubic centimeter and the relative proportion of acid to other types of colonies.

5. Determine the morphology of the organisms making up the colonies of each type and compare with the findings in the microscopical sediment test.

6. Are all organisms present microscopically? Explain your results and draw conclusions.

B. MICROSCOPIC SEDIMENT TEST

Method. 1. Mix the milk well and warm about 30 c.c. to 60° to 70° C.

2. Place 10 c.c. of this well-mixed, warmed milk into each of two sedimentation tubes.

3. Place one tube on each of the scale pans and balance by adding more milk to the lighter tube. The tubes must be equal in weight or they will throw the centrifuge "off center."

4. Centrifuge in a machine designed for this purpose for five minutes, till a more or less considerable compact sediment separates out.

5. Pour or pipette off the milk above the sediment.

6. Fill the tubes with physiological salt solution and mix the sediment well throughout the dilution fluid with a platinum needle.
7. Balance the tubes and centrifuge again.
8. Pour or pipette off the physiological salt solution.
9. With a small platinum loop, obtain a small amount of the sediment and make a smear on a clean slide.
10. Stain with aqueous-alcoholic methylen blue.

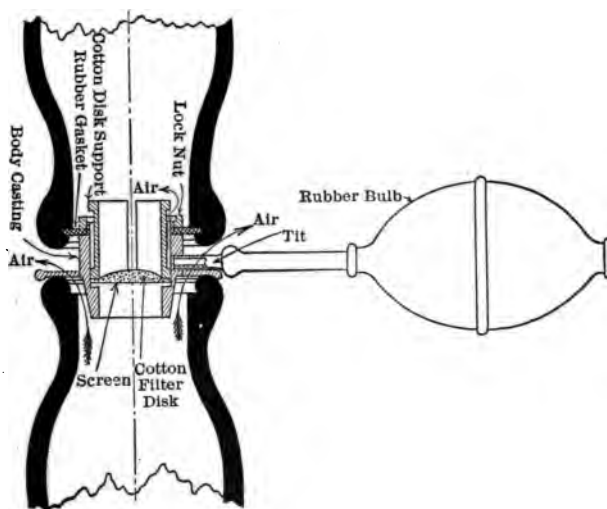


FIG. 60.—Wizard Sediment Tester for Milk.

11. Determine the proportions of bacteria and leucocytes in ten fields. Also note the presence of bacteria in clumps and foreign matter.

The presence of many leucocytes and streptococci associated together is generally indicative of an inflamed condition of the udder, as in mastitis (garget). On the other hand, sometimes the milk from normal udders may show a considerable quantity of leucocytes in the sediment.

C. MACROSCOPIC SEDIMENT TEST

Method. 1. Put a cotton disk in place in the pneumatic sediment tester, heat the sediment tester and clean empty milk bottle in steam thirty minutes, and allow to cool.

2. Attach the sediment tester to the top of the milk bottle containing the sample of milk, using "aseptic" precautions, and invert the whole apparatus over the mouth of the sterile empty milk bottle.

3. Pump the contents of the upper bottle into the lower bottle by means of the rubber bulb. The milk is forced through the cotton disk and leaves its larger particles of insoluble dirt on the cotton.

4. Note the quality of the milk tested by this method. Is there any interrelationship between microscopic sediment test, and the macroscopic sediment test?

5. What does the presence of visible dirt on the cotton indicate? Is this sediment test an argument for straining milk before it goes to the consumer? Is it an argument for running milk through a milk clarifier before putting it on the market?

6. Immediately after straining, plate the milk in litmus lactose agar, using dilutions 1 : 100, 1 : 10,000 and 1 : 1,000,000 as before.

7. Incubate the plates for five days at 25° C. and count, estimating total average number and proportions of types as in A.

8. Compare the counts with those of A, also the proportions of the various types.

Note. This method was formerly used for obtaining an estimate microscopically of the numbers of bacteria in milk. It presents difficulties, however, which lead to many technical errors and therefore it cannot be relied upon to give uniform results. The method is valuable, however, for determining something of the sanitary quality of the milk.

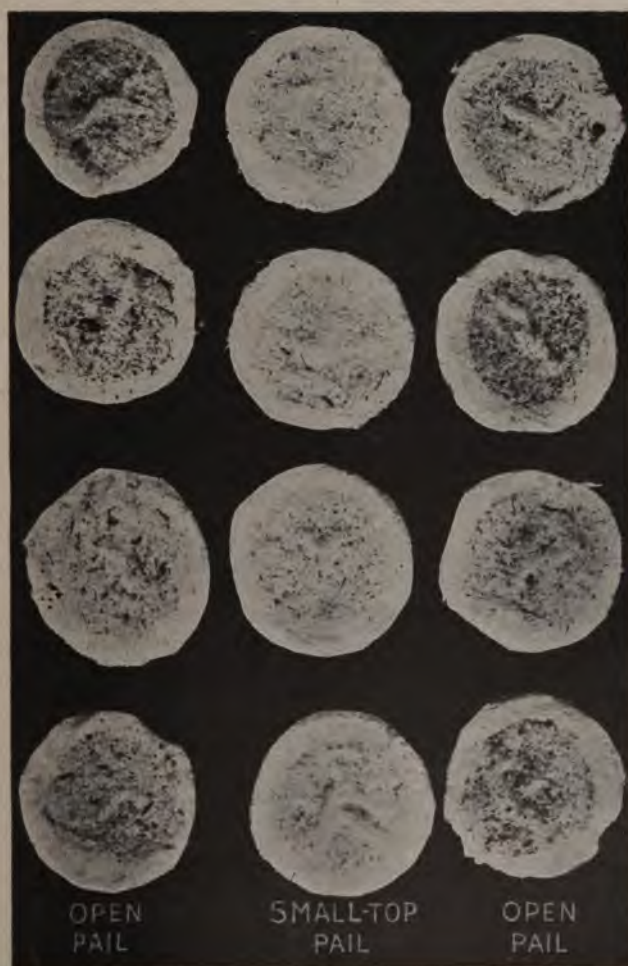


FIG. 61.—Cotton Disks Prepared by the Use of the Wizard Sediment Tester. (Circ. 41, Wisc. Expt. Sta.)

Did straining have any effect on the numbers of organisms present in the milk? What effect may it have? Is this beneficial to the milk as a commercial product?

9. In what way may the microscopic sediment test explain the results obtained by plating milk before and after straining?

10. Make, stain and examine smears from the upper surface of the cotton disk. What is the nature microscopically of the material retained by the cotton? How does this smear compare qualitatively with that from the centrifuged sample?

11. What is the nature of the dirt ordinarily found in milk? How may its presence be eliminated?

12. Give all results in full and draw any conclusions permissible. Point out any practical applications.

REFERENCES

- LÖHNIS: *Laboratory Methods in Agricultural Bacteriology*, pp. 63-65.
JENSEN: *Milk Hygiene*, transl. by Pearson (1907), pp. 126-127.
MARSHALL: *Microbiology*, pp. 326-327.
ROSENAU: *The Milk Question* (1912), pp. 55-88.
ERNST: *Milk Hygiene*, transl. by Mohler and Eichhorn (1914), p. 182.

EXERCISE 5. TO DETERMINE THE INFLUENCE OF TEMPERATURE UPON THE KEEPING QUALITY OF MILK; PURE MILK COMPARED WITH MARKET MILK

One of the most important considerations in the production of milk, either for factory use or for town or city supply, is the temperature at which the milk is maintained. The beneficial effects of scrupulous cleanliness in the production of milk will be largely counteracted unless the milk is cooled immediately after drawn and maintained at a temperature too low for development of the bacteria present.

Apparatus. Three sterile 1 liter Erlenmeyer flasks; one sterile 2 liter Erlenmeyer flask; twenty-four sterile Petri dishes; sterile 10 c.c. pipettes; sterile 1 c.c. pipette graduated to 0.1 c.c.; twenty-four tubes of litmus lactose agar; 90 c.c. and 99 c.c. dilution flasks; ice and salt for preparing freezing mixture; fresh milk and bottled milk.

Method. 1. In a sterile 2 liter Erlenmeyer flask place about $1\frac{1}{2}$ liters of milk from a can of milk immediately after it has been filled by the milkers.

Note. This exercise is to be repeated, for purposes of comparison, using three pint bottles of milk all obtained at one time from the same milkman. In this case the first plating is to be made from each separate bottle.

2. Record the temperature. Plate from the sample immediately in litmus lactose agar, making dilutions of 1 : 100 and 1 : 500. Determine the acidity of the sample, using a sterile 5 c.c. pipette to obtain the sample.

Note. Portions for acidity determination and plating should be removed with sterile pipettes in all instances.

3. Transfer the sample "aseptically" into the three 1 liter flasks, placing an equal portion as nearly as possible in each flask. Label the flasks *A*, *B*, *C*.

4. Cool flask *A* in a freezing mixture to 10° C., and set away in refrigerator to maintain the low temperature.

5. Cool flask *B* in a freezing mixture to 10° C., then place it at a constant temperature of 21° C.

6. Place flask *C* at a constant temperature of 21° C.

7. At the end of twenty-four hours determine and record the acidity of each of the three portions of the original sample.

8. Plate in litmus lactose agar, using the following dilutions:

Flask *A*, 1 : 100 and 1 : 1,000.

Flasks *B* and *C*, 1 : 10,000 and 1 : 1,000,000.

9. At the end of another twenty-four hours repeat the titrations and platings with all flasks, using the following dilutions:

Flask *A*, 1 : 10,000 and 1 : 1,000,000.

Flasks *B* and *C*, 1 : 1,000,000 and 1 : 100,000,000.

10. At the end of five days determine and record the acidity of the milk in all flasks. Plate from flask *A* only, using dilutions of 1 : 1,000,000 and 1 : 100,000,000.

11. All plates should be held at 21° C. for a period of five days before counting.

12. Compare the relative kinds and numbers of colonies in plates from the three flasks. Note also the time of curdling and the nature of the curd formed in each case.

13. Compile the results of the investigation in tabulated form. Plot bacterial count and acidity curves.

14. From the results obtained, what conclusions would you draw as to the influence of cooling upon the keeping quality of milk?

How does the age and original quality of the milk effect its keeping qualities when subjected to different temperature conditions?

How does cooling milk and keeping it cool compare with merely cooling and then allowing the milk to acquire the temperature of the room? What is the explanation of the action occurring?

What is the purpose of cooling the milk as soon as it comes from the cow? What different methods are used? What are some of the disadvantages of the different methods used for cooling?

What bacterial action takes place in the refrigerator milk? Is the germicidal action of milk sufficiently important to recommend a change in the general practice of cooling milk?

15. Give your results in detail and point out any practical applications or conclusions.

REFERENCES

- WARD: Pure Milk and the Public Health (1909), pp. 15-16, 24-25, 37, 121.
 RUSSELL and HASTINGS: Outlines of Dairy Bacteriology, pp. 54-56.
 ERNST: Milk Hygiene, transl. by Mohler and Eichhorn (1914), pp. 148-149, 156.
 MARSHALL: Microbiology, pp. 318-319.

EXERCISE 6. A STUDY OF THE PASTEURIZATION OF MILK OR CREAM BY LABORATORY METHODS

Apparatus. Water bath; test-tube rack of metal to fit water bath; sterile, large tubes selected for uniformity in diameter (2 cm.); sterile Petri dishes; sterile 1 c.c. pipettes, graduated to 0.1 c.c.; sterile litmus lactose agar tubes.

Method. 1. Secure milk or cream, about 125 c.c. to be used for tubing and pasteurizing.

Note. If time permits, it is desirable to test pasteurization upon:

- a. Fresh milk or cream.
- b. Milk or cream which has stood for twenty-four hours but is still sweet.
- c. Milk or cream which has reached an acidity of about 22°.
- d. Milk or cream from different sources, supposedly having different bacterial contents.

2. Tube the sample or samples of milk or cream, pouring 10 c.c. into each tube, filling fifteen tubes for each sample.

Note. Only one sample should be pasteurized at a time.

3. Prepare one tube from each sample of milk or cream for the introduction of the thermometer. By so doing, the conditions practically identical, the temperature will be easily read and controlled.

4. After the tubes are prepared mark tubes in duplicate as follows: 50°, 60°, 70°, 80°, 90° and 100°, leaving two unmarked as controls.

5. Place them in the rack so that the marks on the tubes

may be easily recognized, and insert the rack in the water-bath.

6. Pour water into the water-bath until the height of the water corresponds to the height of the milk in the tubes.

7. Put aside two tubes of milk or cream from each sample, one to be employed for comparative check-observation, and the other for check-plating against those which will be subjected to pasteurization.

8. Apply heat to the water-bath.

9. At 50°, 60°, 70°, 80°, 90° and 100° C., remove two tubes of each sample of milk or cream undergoing pasteurization and place in cold water.

10. Employ one of the tubes thus removed for plating and the other place at a temperature of 25° to 28° C. along with the previous check-observation tube (7).

11. Make two plates in litmus lactose agar from the tube held for check-plating (7) and from one of the two tubes removed at each of the temperatures designated above. The remaining tube is to be left undisturbed and placed at 25° C., to observe macroscopical changes.

Dilutions for plating:

Fresh milk, unpasteurized, 1 : 10 and 1 : 100.

Milk twenty-four hours old, but sweet, unpasteurized, 1 : 10,000 and 1 : 1,000,000.

Milk with an acidity of 22°, unpasteurized, 1 : 100,000 and 1 : 10,000,000.

Milk pasteurized at 50° C. (fresh) 1 : 10 and 1 : 100.

Milk pasteurized at 50° C. (old) 1 : 10,000 and 1 : 1,000,000.

Milk pasteurized at 60° C., 1 : 10 and 1 : 100.

Milk pasteurized above 60° C., 1 : 10.

12. Keep the plates at 25° C. for seven days, counting colonies at the end of this time.

13. Determine the character of the microorganisms left after pasteurization with those before pasteurization

as to the relative number of each kind, to the fermentation of milk or cream, to spore formation, and to resistance. Which microorganisms have succumbed to pasteurization at different temperatures and which were able to withstand it?

14. Record the results obtained from the study of plates and cultures made from colonies.

15. Record your observations from day to day of macroscopical changes in the pasteurized and unpasteurized control tubes. Does pasteurization destroy organisms that are favorable, or detrimental to the milk? What influence does pasteurization have upon the keeping quality of milk?

16. What influence do the following factors have upon the efficiency of pasteurization: the age of milk? acidity? degree of temperature to which milk is subjected? duration of pasteurization temperatures? presence or absence of air? pressure, whether atmospheric or greater? viscosity or other changes in milk or cream?

What changes are accomplished by pasteurization? Why is milk pasteurized? Is this end always accomplished in commercial plants?

What different methods are used commercially for the pasteurization of milk? What are the advantages and disadvantages of each method? Why?

At what stage in the process of production should milk be pasteurized to accomplish the desired results? Must the after-treatment of pasteurized milk be any different from that of unpasteurized milk?

Do you think that milk should be pasteurized before it reaches the consumer?

Does pasteurization affect the digestibility of milk? What are the limitations of pasteurization as applied to milk?

17. Give your results in full and any conclusions that may be drawn.

REFERENCES

- MARSHALL: Microbiology (1911), pp. 319-321.
ROSENAU: The Milk Question (1912), pp. 16, 37, 76, 105, 112, 120, 128, 132, 138, 161, 185-230, 294.
WARD: Pure Milk and the Public Health (1909), pp. 71, 73, 74, 114-125.

EXERCISE 7. DETERMINATION OF THE NUMBER AND TYPES OF BACTERIA IN BUTTER

Apparatus. Three tubes litmus lactose agar; litmus milk tubes; fresh butter; sterile dilution flasks; three sterile Petri dishes; sterile 1 c.c. volumetric (bulb) pipettes.

Method. 1. Melt a small quantity of butter in a test tube at the *lowest possible temperature* (not higher than 40° to 45° C.). Mix well.

2. Using a warm pipette, transfer 1 c.c. of the well-mixed melted butter to 99 c.c. of sterile (warm) salt solution. Free the pipette from fat by filling it with the dilution water several times. Use warm (50° C.) pipettes and dilution flasks throughout so that the butter will not stick to the pipettes and may be readily emulsified.

3. Plate in litmus lactose agar, using dilutions 1 : 1,000, 1 : 100,000 and 1 : 1,000,000.

Note. These dilutions may have to be changed. Look up the average number of bacteria in the type of butter you are using and make dilutions accordingly.

4. Incubate the plates at 25° C.

5. Weigh 1 c.c. of well-mixed melted butter and record the weight in grams.

6. Examine the plates after three to five days for acid and other types of colonies.

7. Count and record the number of bacteria per cubic centimeter, also the types. Note the action of each type on litmus milk.

8. Estimate the number of bacteria per gram.

9. What is the melting-point of butter? Are bacteria ordinarily killed at this temperature?

What kinds of microorganisms are found in fresh butter from ripened cream? In old butter? In fresh oleomargarine? In renovated butter? In canned butter?

Do bacteria increase or decrease in butter kept in storage? What other methods of making a bacteriological examination of butter may be employed?

Are microorganisms in any way responsible for the flavors of butter? Explain.

What pathogenic organisms may gain entrance to butter?

What is the avenue of entrance? How long can bacteria exist in butter? How do bacterial numbers and types compare with those of fresh milk? of ripened cream?

10. Give your data and conclusions in full and point out any practical applications,

REFERENCES

- RUSSELL and HASTINGS: Practical Dairy Bacteriology, pp. 95, 97.
LÖHNIS: Laboratory Methods in Agricultural Bacteriology, pp. 81-82.
MARSHALL: Microbiology, pp. 335-345.
SAYER, RAHN and FARRAND: Keeping Qualities of Butter, I. General Studies, Tech. Bul. I (1908), Mich. Agrl. Expt. Sta.
RAHN, BROWN and SMITH: Keeping Qualities of Butter, II and III. Tech. Bul. 2 (1909), Mich. Expt. Sta.
WELLS, LEVI: Renovated Butter: Its Origin and History. Yearbook of Dept. of Agr. for 1905, pp. 393-398.
ROGERS, L. A.: Studies Upon the Keeping Quality of Butter, I. Canned Butter. Bul. 57, B. A. I., U. S. Dept. of Agr. (1904), pp. 6-8, 22-23.

EXERCISE 8. TO DETERMINE THE NUMBER AND TYPES OF MICROORGANISMS IN CHEESE

Apparatus. Cheddar cheese; cheese trier (sterile) if an uncut cheese is to be sampled; mortar, with pestle; two knives, sterile; quartz sand; sterile filter papers about 6 and 8 cm. square; one dilution flask containing 95 c.c. of 0.85% salt solution; dilution flasks, containing 90 and 99 c.c. sterile 0.85% salt solution; sterile 1 c.c. and 10 c.c. pipettes; three tubes litmus lactose agar; three sterile Petri dishes; sterile litmus milk tubes.

Method. 1. Sterilize in the hot-air oven, 10 gms. of sand in a mortar with a pestle.

2. Using a red-hot knife blade, sear a portion of the surface of the cheese.

3. To weigh the cheese "aseptically," place the smaller sterile filter paper upon the larger on the balance pan.

4. With a sterile knife remove the inner portion of the seared surface and obtain and weigh out 5 gms. of cheese, using aseptic precautions.

5. Transfer the cheese to the sterile mortar and grind up well.

6. Transfer the cheese and sand mixture with sterile knife or spatula to the 95 c.c. dilution flask. Shake well to free the sand from the cheese.

Directions for making dilutions. In transferring with a pipette a portion of the first suspension to other dilution flasks the sample should be taken immediately after shaking before the sand has settled. Settling may be avoided by holding the pipette in a horizontal position until ready to deliver the contents. The grinding material should be fine enough to avoid clogging the pipette.

7. Make and plate the following dilutions in litmus lactose agar: 1 : 100,000; 1 : 10,000,000 and 1 : 1,000,000,-000, if the cheese has been made recently.

If the cheese is not perfectly fresh, use dilutions 1 : 100,-

000; 1 : 1,000,000 and 1 : 10,000,000. Lower dilutions may be necessary if the cheese has been stored for some time.

8. Incubate the plates at 25° C. for three to five days.

9. Count and estimate the number of microorganisms per gram of cheese. What types predominate? Why?

10. Transfer the different types to litmus milk and note the action after several days. Which of these types, as determined from the action on litmus milk, may have a prominent part to play in the ripening of the cheese? Why?

12. Do microorganisms play any part in the formation of the flavor of cheddar cheese? Other cheeses?

How does the cheese analyzed compare with the butter analyzed as to numbers and types of microorganisms?

What qualitative tests are made for milk used for cheese making? What is the principle and application of these tests?

What cheese "abnormalities" may be caused by microorganisms? During what stages in the process of making cheese may these occur?

What pathogenic organisms have been found in cheese? What is known of their longevity in this medium?

13. State your results and conclusions in full and point out any practical applications.

REFERENCES

- LÖHNIS: Laboratory Methods in Agricultural Bacteriology, pp. 83-88.
 RUSSELL and HASTINGS: Experimental Dairy Bacteriology, pp. 103-109.
 MARSHALL: Microbiology, pp. 346-357.
 CONN: Practical Dairy Bacteriology (1907), pp. 223-259.
 SAVAGE: The Bacteriological Examination of Food and Water (1914), pp. 118-119.
 DECKER: Cheese Making (1905), pp. 48-51, 63, 66, 69, 75, 88, 108-110.
 VANSLYKE and PUBLOW: The Science and Practice of Cheese-making (1912), pp. 115-135, 285-312, 371-378.

EXERCISE 9. A COMPARISON OF THE BACTERIAL CONTENT OF SWEETENED AND UNSWEETENED CONDENSED MILKS

Apparatus. Six sterile Petri dishes; six tubes of litmus lactose agar; 99 c.c. dilution flasks; two 95 c.c. dilution flasks; tubes of sterile litmus milk; two sterile 5 c.c. pipettes with large aperture for delivery; can-opener.

Culture. Unopened can of sweetened condensed milk; unopened can of unsweetened condensed milk (contents guaranteed to be sterile).

Method. 1. Sterilize the can-opener in the flame.

2. Thoroughly cleanse the outside of the unopened cans of condensed milk and then submerge in boiling water for five or ten minutes.

3. Remove the cans from the water, being careful in handling them not to contaminate the upper surface of the cans.

4. With the sterile can-opener make an opening in the can only large enough to admit the introduction of a 5 c.c. pipette.

Note. Only one can is to be opened at a time to avoid contamination.

5. With a sterile pipette obtain a 5 c.c. sample from the can just opened and transfer to a 95 c.c. dilution flask.

Note. As the condensed milk is very viscous and adheres to the sides of the pipette, after delivering the 5 c.c. into the dilution flask blow out the remainder into the sink or other suitable place, then replace in the dilution flask and wash out the adhering fluid by drawing the diluting fluid up into the pipette several times. The use of a 5 c.c. volumetric pipette having a large aperture for delivery would lessen the possibilities of contamination.

6. This resulting dilution is a 1 : 20 dilution of the condensed milk, or a 1 : 40 dilution of the original milk (if the directions on the can give a dilution of 1 : 1 for producing a milk of original composition).

7. Plate the following dilutions of the condensed milk in litmus lactose agar: 1 : 20, 1 : 2000 and 1 : 20,000. Place plates at 25° C.

8. Examine and count at the end of five days.

9. Record the numbers and types of organisms developing on the plates. Are any acid colonies present? Determine the morphology of the acid colonies.

10. Transfer each type of colony to a tube of sterile litmus milk and observe action from day to day. Are the types which are present desirable? Is *Bact. lactis acidi* present? Any organisms of the *B. coli* type? Are pathogenic bacteria apt to be present?

11. To what factors are due the keeping qualities of each type of condensed milk?

What care should be taken of opened cans of milk of either type? Of the milk after it has been diluted according to directions?

In what other forms is concentrated milk sold? What factors are responsible for the keeping quality of each of these latter types?

12. Give your results and conclusions in detail.

REFERENCES

MARSHALL: Microbiology (1911), 363-366.

SAVAGE: The Bacteriological Examination of Food and Water (1914), pp. 111-113.

SADTLER: Industrial Organic Chemistry (1912), pp. 281, 288.

EXERCISE 10. TO DETERMINE THE NUMBER AND TYPES OF MICROORGANISMS IN ICE CREAM

Apparatus. Litmus lactose agar shake; three tubes sterile litmus lactose agar; three sterile Petri dishes; sterile 1 c.c. pipettes; sterile dilution flasks; sterile wide-mouthed glass-stoppered bottle; sterile butter trier; sterile knife.

Culture. From ice cream.

Method. 1. Remove the (frozen) ice cream sample from the container by means of the sterile butter trier.

2. With the sterile knife discard the upper portion of the sample and place in the sterile wide-mouthed bottle.

Note. Pack the sample in ice if it cannot be examined at once.

3. To examine, allow the ice cream to melt quickly by placing it at about 37° C. and then treat as a milk sample.

4. Plate on litmus lactose agar, using the following dilutions: 1 : 10,000, 1 : 1,000,000 and 1 : 100,000,000 and incubate plates at 37° C.

5. Add a large quantity (25 c.c. to 50 c.c.) to the melted agar shake and incubate at 37° C. Examine in twenty-four to forty-eight hours for acid and gas. Is *B. coli* present?

6. Count plates at the end of three days and estimate the total number of bacteria present per cubic centimeter, also the number of acid colonies and of any other predominant type.

7. Transfer predominant types to litmus milk tubes and note action, also note rapidity with which each type produces changes in the litmus milk. What may these results signify?

8. Make a microscopic count, using the method in Exercise 1, Dairy Microbiology. How do microscopic and plate counts compare?

9. Look up references for ascertaining bacteriological standards for ice creams. What is the quality of the ice cream you analyzed as compared with the maximum bacterial limit? What do you think this limit should be?

10. From what diverse sources do bacteria enter ice cream?

What is their significance in this product?

What relation may some of the common practices of ice-cream makers have to the bacterial content of milk?

What effect does storage have upon the number of bacteria in properly hardened ice cream?

What significance has a pure ice-cream supply in relation to public health?

11. Give results and conclusions in detail.

REFERENCES

- SAVAGE: The Bacteriological Examination of Food and Water (1914), pp. 119-121.
 MARSHALL: Microbiology (1911), pp. 372-373.
 WILEY, H. W.: Ice cream, Hygienic Lab. Bul. 56. Milk and its Relation to the Public Health (1909), pp. 251-311.
 HAMMER, B. W.: Bacteria and Ice Cream, Bul. 134 (1912), Iowa Agr. Expt. Sta.
 MORTENSEN, M. and GORDON, J.: Lacto: a New and Healthful Frozen Dairy Product. Bul. 119 (1911), Iowa Agr. Expt. Sta.
 WASHBURN, R. M.: Principles and Practice of Ice-cream Making. Bul. 155. Vermont Agr. Expt. Sta., pp. 9-10, 34-46, 53-54, 64-66.
 BOLDUAN: Food Poisoning (1909), pp. 84-90.
 AYERS, S. H. and JOHNSON, Jr., W. T.: A Bacteriological Study of Retail Ice Cream. Bul. 303 U. S. Dept. Agr., 1915.

PLANT MICROBIOLOGY

EXERCISE 1. TO DEMONSTRATE THAT PLANTS ARE SUBJECT TO MICROBIAL DISEASES: INFECTION OF CERTAIN SPECIES OF VEGETABLES HAVING JUICY ROOTS, LEAVES, FRUITS, ETC., WITH *B. CAROTOVORUS*

Apparatus. Tubes of sterile 2% saccharose broth; tubes of sterile agar; sterile water; sterile Petri dishes; three sterile deep culture dishes; sterile filter paper; sterile knife; sterile forceps; mercuric chloride, 1 : 500; juicy vegetables.

Culture. *B. carotovorus* (culture of high physiological efficiency).

Method. 1. The root of the carrot, turnip, rutabaga, the cucumber or radish; the cotyledons of immature pea

seedlings, petioles of cabbage seedlings, potatoes, etc., may be used for this exercise. For what other plants is *B. carotovorus* pathogenic?

2. Thoroughly wash the root, or vegetable to be in-



FIG. 62.—Crown Gall Produced by *Bact. tumefaciens*. (Orig.)

oculated. Two or three vegetables of one kind should be employed.

3. Disinfect a spot about 2 cm. in diameter with 1 : 500 mercuric chloride and rinse with sterile water to get rid of disinfectant. Drain off excess moisture on sterile filter paper, handling vegetable with sterile forceps.

4. Puncture the disinfected area on one vegetable with

the sterile stiff needle for control and place in a sterile deep culture dish.

5. Obtaining some of the culture of *B. carotovorus* on the sterile needle, puncture the remaining vegetables in the center of the disinfected area and place vegetables in a sterile deep culture dish at 20° to 25° C.

B. carotovorus is a wound parasite which invades the intercellular spaces, dissolving the middle lamellæ and portions of the inner lamellæ, thereby establishing a condition which is known as soft rot.

6. Examine in twenty-four hours for evidence of action of *B. carotovorus*. This should be easily distinguishable in three days.

7. Isolate the causal organism and determine its morphology and cultural characteristics. Compare with pure culture and with description given in Marshall's Microbiology, p. 512.

Is the organism newly isolated, capable of producing infection? Make inoculations from one, two, three and four-day old newly isolated cultures to sterile living vegetable tissue to determine this. Is there any difference in the infectivity of a one-day old and a three- or four-day old culture?

8. What is known of methods of control of this disease?

9. Grow four giant colonies of *B. carotovorus* on ordinary agar, one in each Petri dish and allow them to develop until nearly 1 cm. in diameter.

10. Under sterile conditions, remove slices of fresh carrot, beet and rutabaga or turnip roots and potato and place in sterile Petri dishes. (Slices should be at least 3 to 4 cm. in diameter.)

11. With a sterile scalpel make a circular incision 0.5 cm. from the edge of the colony through the layer of agar in the Petri dish.

12. Remove this colony intact to the surface of one of the slices of vegetable and replace cover of Petri dish.

13. Repeat, removing a colony to the slice of each of the different vegetables.

14. Examine in twenty-four hours for evidences of soft rot, and note progress of softening from day to day. What is demonstrated by this phenomenon? Are all vegetables attacked?

15. What parts of the plant does *B. carotovorus* attack? What chemical constituents of these parts are decomposed through the agency of their action?

What are the main features of difference in the mechanism of action of the various types of bacterial plant diseases?

Give an example of a disease illustrating each.

How is the progress of infection effected in these various types? What organisms produce a disease of similar type in other vegetables and plants?

What is known of immunity in the plant kingdom?

What methods of control are employed with different types of plant diseases? How are methods of control influenced by the type of disease?

Note. This exercise may be made more interesting and instructive if combined with histological methods.

Plates illustrating the invasion of root tissues by *B. carotovorus* are found in Smith's *Bacteria in Relation to Plant Diseases*, Vol. I, pp. 56, 103.

16. State in full your results and conclusions.

REFERENCES

- SMITH, ERWIN F.: *Bacteria in Relation to Plant Diseases*, Vol. I, pp. 5, 6, 65, 86, 103. Vol. II, pp. 51-52, 65, 81-88, 96, 292. Vol. III.
- MARSHALL: *Microbiology*, pp. 490-519.
- JONES, L. R.: A soft rot of carrot and other vegetables, pp. 299, 13th Rept. of Vt. Expt. Sta. (1901). Also in *Cent. f. Bakt.* II, Bd. 14, pp. 369-377.
- JONES, L. R.: Pectinase, the cytolytic enzyme produced by *B. carotovorus* and certain other soft-rot organisms. *Tech. Bul.* 11, N. Y. Agr. Expt. Sta. (1909).

ANIMAL DISEASES AND IMMUNITY

EXERCISE 1. ANIMAL INOCULATION IN BACTERIOLOGY FOR DETERMINATION OF THE IDENTITY OF A MICROORGANISM, ITS PATHOGENICITY OR VIRULENCE, OR FOR PRODUCTION OF IMMUNITY

Apparatus. Experimental animals: rabbits; guinea pigs; white rats; white mice, etc.; scalpels; scissors; forceps; razor; syringe; trephine; sterile dishes; anesthetic; disinfectant; cotton.

Culture. Pure culture or infected material.

I. INTRODUCTION

1. Avoid the use of animals where the employment of other means answers the purpose equally well.

2. Unless other factors prevent, always use the most susceptible and least expensive animals.

II. PREPARATION OF ANIMAL *

Method. 1. (a) Examine carefully each animal before subjecting it to experimentation.

(b) Use no animal already showing symptoms of illness or general lack of vigor.

(c) Record the weight and temperature of each animal.

2. (a) Administer an anesthetic (general or local as indicated) whenever the operation is very painful or tedious or where perfect immobility of the parts is required.

Note. For local anesthesia a 2% solution of cocaine hydrochloride may be made by dissolving 0.1 gm. of cocaine hydrochloride in 5 c.c. of sterile water. Instill a few drops into the conjunctival sac or inject 1 to 5 c.c. into the subcutaneous tissues near the seat of operation. For general anesthesia 10 to 30 c.c. of a 5% solution of chloral hydrate may be injected per rectum, or ether or chloroform may be inhaled.

*The instructor must arrange for experiments that must be started early in order to be completed before the term closes.

Ether is probably safer in the hands of a novice. It may be administered by saturating cotton placed in a paper cone which is kept over the animal's nose. Care should be exercised to replenish the supply of the anesthetic on the cotton as fast as it volatilizes and not to force the anesthetizing too fast. Injury to the integument about the nose may be avoided by rubbing on vaseline before beginning the operation. The tissues should not be cut until anesthesia is complete.

- (b) Choose a site for operation where the results will not interfere with the animal's locomotion or normal functions.
- (c) Use sharp, sterile instruments.

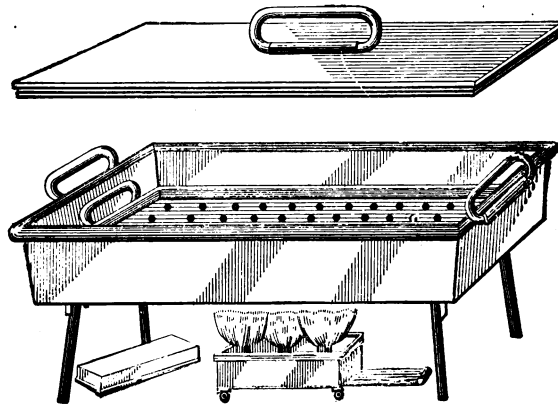


FIG. 63.—Tray for Sterilizing Surgical Instruments.

Note. Methods for holding different animals for different forms of operations vary. An assistant is usually required to hold the animal, where an anesthetic is not administered, and where an anesthetic is used it is usually better to have an assistant administer it, although this is not necessary. (For various devices for holding experimental animals see text-book: Eyre, *Bacteriological Technic*, 2d Ed. (1913), pp. 349-352.)

3. Remove the hair with scissors or clippers from the field of operation and shave the surface. Wash the skin and disinfect it with 2% liquor cresolis compositus (U. S. P.). Wash off the disinfectant with alcohol and allow the

alcohol to evaporate. The animal is now ready for the operation.

Note. It is understood that a 2% solution of liquor cresolis compositus (U. S. P.) shall be used wherever a disinfectant solution is indicated unless otherwise stated.

III. METHODS

Where the exact nature of the inoculum is unknown, the experimenter will be guided, as to what method to select, by his judgment, influenced by experience with other inocula in animal experimentation. The method most adaptable in the case of each specific microorganism will be indicated in the treatment of that organism.

1. *Cutaneous.* Rub the inoculum on the shaved and disinfected skin or make several parallel, superficial incisions and rub the inoculum into the scarifications with a sterile scalpel. See that no disinfectant remains on the skin before operating.

2. *Subcutaneous.* I. (a) Pick up the skin with the thumb and forefinger of the left hand and insert the needle through one side of the fold of skin thus made.

Note. The point of the needle should not enter the skin on the other side of the fold, but should lie in the subcutaneous tissue.

(b) Release the skin and inject the material.

(c) Place the finger moistened with the disinfectant over the point where the needle enters the skin and remove the needle.

II. (a) For solid material that will not pass through a hypodermic needle, make a short incision through the skin parallel to the horizontal plane of the body.

(b) With a sterile probe separate the skin from the underlying tissues on the lower side of the cutaneous incision, making a small pocket in the subcutaneous tissue.

(c) With fine-pointed sterile forceps insert the inoculum

into this pocket. Further treatment should not be necessary.

3. Intramuscular. I. Plunge the needle deeply into the muscles, preferably on the inside of the thigh.

II. Inject the material slowly with steady pressure if the volume is great.

4. Intravenous. I. Inject the liquid into the ear vein



FIG. 64.—One Method of Injecting Hog-cholera Serum. (Orig.)

of the rabbit (and other animals if possible) or jugular vein where accessible. The injection should be in the direction of the circulation.

II. The femoral vein may be used where other veins are not readily entered with the needle. Use general anesthesia. Make an incision on the inside of the thigh over the femoral space. Separate the iliacus, pectineus and sartorius muscles. The femoral vein and artery are laid bare. After inoculation disinfect and suture the skin.

Note. Solid substances, larger than leucocytes, and air bubbles should not be injected into the vascular system. Fatal emboli may result. Return of blood through the needle indicates that the vein has been entered. If swelling occurs at point of inoculation the inoculum is entering the subcutaneous tissue. Try again.

5. Intraabdominal or intraperitoneal. I. The site for the operation is the center of the angle formed by the last rib, transverse processes of the lumbar vertebræ, and the external angle of the ilium.

(a) Plunge the needle or trocar and canula through the abdominal wall with one thrust.

Note. When the parietal peritoneum is punctured the sudden disappearance of resistance to the entrance of the needle is noticed. The intestines will not be entered if pressure on the needle stops at this point.

(b) Inject the material and remove the needle, placing the thumb and finger on each side of the needle and pressing gently on the skin during the removal so as to prevent separating the skin and underlying layers of tissue.

II. Infectious material or cultures in a sterile collodium capsule may be introduced into the abdominal cavity by performing laparotomy. (General anesthesia is desired.)

6. Intraorbital. Always perform under local anesthesia. (2% cocaine hydrochloride.)

I. Steady the eye with fixation forceps.

II. Pierce the cornea near to its periphery with a fine needle. The needle should incline with the point outward so that, upon entering the anterior chamber of the eye, the iris will not be damaged.

III. Inject the material.

7. Subdural. Operate under general anesthesia.

I. Make a longitudinal incision through the skin at one side of the sagittal suture. Hold back the skin and subcutaneous tissue with tenacula.

II. Make a crucial incision through the periosteum and push back the four corners.

into this pocket. Further treatment should not be necessary.

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III. Inject the material.

7. Subdural. Operate under general anesthesia.

I. Make a longitudinal incision through the skin at one side of the sagittal suture. Hold back the skin and subcutaneous tissue with tenacula.

II. Make a crucial incision through the periosteum and push back the four corners.

III. Expose the dura mater by removing a small button of the parietal bone (0.5 cm. in diameter) with a trephine.

IV. Inject the inoculum immediately beneath the dura mater.

V. Replace the periosteum and suture the skin.

VI. Disinfect.

8. *Intrapulmonary.* I. Pull the animal's front leg forward.



FIG. 65.—Another Method of Injecting Hog-cholera Serum. (Orig.)

II. Plunge the needle through the fifth or sixth intercostal space into the lung tissue.

III. Slowly inject the contents of the syringe.

Note. In large animals material may be injected into the trachea between the tracheal rings.

9. *Ingestion.* If possible mix the infectious material with the animal's food.

Note. See that the animal eats all that is intended to be eaten.

Introduce the infectious material into a gelatin capsule and force the animal to swallow it; or give the material as a drench where advisable.

Note. Fasting the animal before introducing unpalatable material into the food may be helpful in increasing the amount eaten. The chemical reaction of the stomach contents as governed by physiological activity will influence results.

IV. CARE OF INOCULATED ANIMALS

1. Watch each animal closely and take temperatures as the case demands.

2. Treat each animal as a case of infectious disease in quarantine.

Note. Whatever clinical, diagnostic or sanitary measures necessary in that given disease may be employed as seen fit.

3. When the animal is removed from the cage for the last time, carefully destroy all refuse in the cage and disinfect thoroughly.

4. Give all results, observations and conclusions in detail.

REFERENCES

STITT: Practical Bacteriology, Blood Work and Parasitology, 3d Ed., 1914, pp. 48, 143, 152.

MOORE and FITCH: Bacteriology and Diagnosis, pp. 114, 118-120, 124, 125, 130, 133.

EYRE: Bacteriological Technic, pp. 332-369.

KOLMER: Infection, Immunity and Specific Therapy (1915), pp. 53-64.

EXERCISE 2. THE ISOLATION OF PATHOGENIC BACTERIA FROM FLUIDS AND TISSUES OF DEAD ANIMALS

Apparatus. Disinfectant; scalpel; scissors; forceps; bone forceps; ten sterile pipettes; 10 c.c. sterile pipettes; 250 c.c. flask containing glass beads, sterile; sterile Esmarch dishes; spatula; platinum loop; special media.

Method. 1. Disinfect the skin.

2. Remove the spleen, kidney, lymph glands, and any other diseased tissue, to sterile Esmarch dishes, using sterile instruments.

3. Collect samples of pericardial and pleuritic fluids, blood, urine and bile with sterile pipettes and place these in small sterile flasks. Collect at least 25 c.c. of blood in a sterile flask containing glass beads for defibrinating.

4. Remove the organs collected to the laboratory and make cultures as follows:

5. Sear the surface of the organ with a spatula heated to a white heat.

6. Tear the seared surface with forceps, sterilized in flame.

7. With a sterile platinum loop, make transfers to agar slants, shake cultures, and plates for isolation into pure cultures.

8. Repeat 7, using any body fluids collected.

Note. The different diseases require special procedures and media for successful results. Attention will be called to these variations at the proper places.

REFERENCES

MOORE and FITCH: Bacteriology and Diagnosis, pp. 95-96.

MOORE: Principles of Microbiology, pp. 156-162, 237-258.

EYRE: Bacteriological Technic, pp. 248-258.

EXERCISE 3. A STUDY OF BACT. ANTHRACIS

Note. *Bact. anthracis* is the cause of anthrax, a disease very fatal to man and certain domestic animals. Great care should be taken while working with it.

Apparatus. Six tubes of agar; three tubes of potato; three tubes of milk; tube of gelatin; slides and stains; autopsy instruments.

Culture. *Bact. anthracis*.

Method. 1. Inoculate three tubes each of agar, potato, and milk and one tube of gelatin with *Bact. anthracis*.

2. Incubate one tube of each at 20° C., one at 37° C., and one at 42° C. (Study and record the effect of these temperatures upon the growth and spore formation of the organism.)

3. Make cover-glass preparations and stain with methylen blue, fuchsin and Gram's stain. Stain for spores (Anjeszky's method).

4. Transfer a small quantity of the agar culture to 4 or 5 c.c. of sterile physiological salt solution and inject 0.25 c.c. subcutaneously into a guinea pig. Make daily observations and an autopsy of the animal at death.

5. Make cultures on agar slants, and smear preparations from the blood, liver, spleen and kidney after the autopsy.

6. Fix the smears in the flame, stain with methylen blue or fuchsin. After twenty-four and forty-eight hours examine the cultures microscopically.

7. State your results and conclusions in full.

REFERENCES

- MARSHALL: Microbiology, pp. 469, 476, 559, 561, 599-604.
JORDAN: General Bacteriology, 4th Ed. (1914), pp. 223-236.
BESSON: Practical Bacteriology, Microbiology and Serum Therapy, transl. by Hutchens (1913), pp. 517-535.
KOLMER: Infection, Immunity and Specific Therapy (1915), pp. 653-654.
ZINSSER: Infection and Resistance (1914), pp. 15, 18, 53, 64, 296.

EXERCISE 4. THE PREPARATION OF TUBERCULIN

Apparatus. Two 500 c.c. Erlenmeyer flasks; glycerinated veal broth; evaporating dish; 0.5% phenol salt solution; Berkefeld filter; heavy filter paper; 20 c.c. homeopathic vials; sealing wax.

Culture. *Bact. tuberculosis* (specially adapted for tuberculin).

Method. 1. Place about 200 c.c. of glycerinated veal

bouillon in each of two 500 c.c. Erlenmeyer flasks and sterilize for twenty minutes each day for three consecutive days.



FIG. 66.—*Bact. tuberculosis* (avian), on Banana. (Orig. Himmelberger.)

2. From a culture of the tubercle bacterium furnished, inoculate each flask of veal broth. In making the inoculations care should be taken to place the inoculum on the surface and to avoid agitation after inoculation. Seal the flasks with paraffin and place in the incubator at a temperature of 37° C.

3. Allow the cultures to grow four weeks after the surface is covered, then shake well, place in a steam sterilizer and subject to steam for two and a half hours.

4. Filter through a filter paper to remove most of the bacterial growth.

5. Evaporate to one-tenth its original volume over a water bath at a temperature of 60° C.

6. To one volume of the concentrated tuberculin add seven volumes of sterile physiological salt solution containing 0.5% phenol or tricresol and then filter through a Berkefeld filter.

7. Place the product in 20 c.c. homeopathic vials and seal with wax. Label the vials and place in a cool dark room.

REFERENCES

MARSHALL: Microbiology, pp. 485, 487.

MOORE: Principles of Microbiology, pp. 251-253.



FIG. 67.—Glycerin Veal-broth Cultures of *Bact. tuberculosis* (Human), for Tuberculin, about Eight Weeks Old. (Orig. Keck.)

BESSON: Practical Bacteriology, Microbiology and Serum Therapy, pp. 289-345.

MOORE: Bovine Tuberculosis.

KOLMER: Infection, Immunity and Specific Therapy (1915), pp. 582-601, 661-680.

ZINSSER: Infection and Resistance (1914), pp. 355-357, 438-442.

**STATE LIVE STOCK SANITARY COMMISSION
MICHIGAN.**

No. 1057Owner J. S. ReedConsignor W. R. GorkhamFrom Potoskey, Mich.Address Potoskey, Mich.Destination Madison, Wis.Date June 23, 1918

NO.	AGE.	SEX.	DESCRIPTION.	DATE <u>June 22</u>										DATE <u>June 23</u>				REMARKS.		
				TEMPERATURES BEFORE INJECTION										TEMPERATURES AFTER INJECTION.						
				6 A.M.	8 P.M.	10 A.M.	12 M.	2 P.M.	4 P.M.	6 P.M.	8 P.M.	10 P.M.	12 M.	2 P.M.	4 P.M.	6 P.M.				
1	6 yr	Fem.	Grade Holstein	100.8	101.5	101.5	102.	102.	101.5	101.	101.	101.	102.3	102.5	104.	104.	103.7	103.6	102.6	Reactor
100411 yr.	Fem.	Guernsey Heifer	101.8	101.7	101.4	101.1	102.3	101.	101.	101.	101.	102.3	102.5	101.5	101.4	101.2	101.3		Non-Reactor	
3	8 yr	Fem.	Grade Jersey	101.6	101.6	100.5	101.8	101.8	101.8	101.6	101.6	101.6	103.1	102.8	106.2	106.7	106.2	106.		Reactor

107 ——— No. 1

106 - - - - No. 10041

105 - - - - No. 3

104

103

102

101

100

Pre-Injection Curves

Post-Injection Curves

I hereby certify that I have inspected and tested with tuberculin the 3 animals above described, and have found them to be free from tuberculosis 407746
or symptoms of contagious, infectious, or communicable disease 746 examination of Nos. 1 and 3.

Car No. 257A(Signature) John Doe

Veterinarian

Shipped via P.M. and C.M. & S.P.(Approved) Richard Roe

State Veterinarian

EXERCISE 5. THE PREPARATION OF BLACK-LEG VACCINE

Apparatus. Sterile mortar and pestle; sterile cheese-cloth; two sterile glass plates; sterile water; sterile homeopathic vials.

Culture. Diseased muscle of calf affected with black-leg.

Method. 1. Place a piece of diseased muscle from a calf affected with black leg (this will be furnished by the instructor) in a sterile mortar, add a small quantity of water and triturate completely with a sterile pestle.

2. Squeeze the pulp through a piece of sterile cheese cloth, spread the filtrate in a thin layer over a sterile glass plate or saucer and dry at a temperature of 35° to 37° C. in an atmosphere free from contamination.

3. Mix approximately one part by volume of the dried virus with two parts of water, triturate until the mixture is converted into a semi-solid homogeneous mass and spread in a thin layer over a glass plate or saucer.

4. Heat in an oven to a temperature of 100° to 104° C. for a period of seven hours.

5. One centigram of the attenuated virus mixed with a small quantity of water is a dose for a calf. Place the product in sterile vials, ten doses to the vial, place a cork stopper in each vial and seal.

REFERENCES

- NORGAARD, V. A., and MOHLER, J. R.: Black leg, its Nature, Cause and Prevention, B. A. I. Circular No. 31, revised (1911).
MARSHALL: Microbiology, pp. 472-473.
KOLMER: Infection, Immunity and Specific Therapy (1915) p. 655.

EXERCISE 6. THE PREPARATION OF TETANUS TOXIN

Apparatus. Dextrose broth; two 100 c.c. Erlenmeyer flasks; paraffin oil; 5% phenol; Berkefeld filter.

Culture. *B. tetani*.

Method. 1. Place 50 c.c. of dextrose bouillon in each of two 100 c.c. Erlenmeyer flasks, plug and boil gently two or three minutes over a flame.

2. Cover the bouillon with a layer of paraffin oil about 5 mm. deep and heat in the autoclav.

3. After cooling, inoculate the bouillon with *B. tetani* and incubate about two weeks.

4. Examine the culture microscopically to determine the absence of contamination, add sufficient 5% phenol to make a 0.5% solution and filter through a Berkefeld filter.

5. Incubate about 1 c.c. of the filtrate in 10 c.c. of dextrose broth under anaerobic condition, for forty-eight hours to make sure that the filtrate is sterile.

6. The filtrate, if sterile, is to be used in immunizing a rabbit for the production of antitoxin.

Note. A fairly potent toxin will kill a guinea pig in a 0.001 c.c. dose. The toxin in solution is very unstable and should be kept in a tightly stoppered bottle in a cool dark place. It may be kept for several months by precipitating with a saturated solution of ammonium sulphate and drying in vacuo over sulphuric acid.

REFERENCES

- MARSHALL: Microbiology (1911), pp. 480-484.
BESSON: Practical Bacteriology, Microbiology and Serum Therapy (1913), pp. 536-548.
KOLMER: Infection, Immunity and Specific Therapy (1915), pp. 115, 234, 720, 819.
ZINSSER: Infection and Resistance (1914), pp. 41, 107, 131-133.

EXERCISE 7. THE PREPARATION OF TETANUS ANTITOXIN

Note. In the preparation of tetanus antitoxin for therapeutic purposes, healthy horses are used. For the first injection of toxin a small fraction of a cubic centimeter is given subcutaneously. The increase in the size of the dose and the frequency of injection depend upon the condition of the animal, but the quantity injected is gradually increased until the animal is able to stand 300 to 400 c.c. of toxin at one injection.

For laboratory purposes, the rabbit may be used to furnish the antitoxin.

The following method suggested by Roux and Vaillard produces a satisfactory antitoxin for laboratory study.

Apparatus. Tetanus toxin; Gram's iodine solution; rabbits; 20 c.c. syringe; disinfectant; anesthetic; operating tray; 50 c.c. sterile glass cylinder.

Method. 1. Give the first five or six injections subcutaneously, subsequent ones may be given intraperitoneally.

1st day, 3 c.c. of toxin mixed with 1 c.c. of Gram's iodine solution.

5th day, 5 c.c. of toxin mixed with 2 c.c. of Gram's iodine solution.

9th day, 12 c.c. of toxin mixed with 3 c.c. of Gram's iodine solution.

16th day, 5 c.c. of undiluted toxin.

23d day, 10 c.c. of undiluted toxin.

30th day, 15 c.c. of undiluted toxin.

The quantity may be gradually increased until the rabbit is getting 100 c.c. of undiluted toxin.

2. After the 6th injection has been given, wait a period of ten days and bleed the rabbit aseptically. This is accomplished as follows:

(a) Secure the rabbit in a dorsal position on an operating tray and anesthetize with ether.

(b) Expose an area about 3 cm. square over the inferior

thoracic wall, in the region of the apex of the heart, shave and clean with alcohol.

(c) Insert a sterile needle attached to a sterile 20 c.c. syringe, through the thoracic wall into the heart and slowly draw the plunger out.

If only a small quantity of serum is desired for testing, the animal may be saved for subsequent bleedings.

(d) Place the blood in a sterile container, allow to clot and draw off the serum for standardization.

REFERENCES

- MARSHALL: Microbiology (1911), pp. 480-484.
BESSON: Practical Bacteriology, Microbiology and Serum Therapy (1913), pp. 544-548.
KOLMER: Infection, Immunity and Specific Therapy (1915), pp. 234, 242, 719-729.
ZINSSER: Infection and Resistance (1914), p. 463.

EXERCISE 8. A DEMONSTRATION OF THE AGGLUTINATION TEST

Note. There are two methods of applying the agglutination test: First, by combining the suspect's serum in varying amounts with a suspension of the specific organism and incubating eighteen to thirty-six hours; the results are then read with the unaided eye. Second, the serum may be combined in varying dilutions with a suspension of the specific organism, and hanging drop preparations made and examined microscopically. If agglutinins are present, clumping of the organisms will occur in a few minutes. With either method, controls, containing the organism but normal serum, should be prepared for comparative purposes.

Apparatus. Four agar slants; test-tube rack for small test tubes; twelve small test tubes; antiserum; physiological salt solution; 1 c.c. pipettes, graduated to 0.01 c.c.; 5 c.c. pipettes; cover-glasses; concave slide.

Culture. *B. typhosus* or *B. cholerae suis*.

Method. *Macroscopic Test.* 1. *Antigen.* This is a suspension of the specific organism obtained from a twenty-four to forty-eight hour agar culture in physiological salt

solution. Only a sufficient quantity of the growth to give a slight cloudiness to the salt solution in a small test tube should be used.

Note on Antigen. Where a series of agglutination tests are to be made at intervals, the antigen should be standardized so that the same concentration will be used for each test. Great care should be used in preparing the antigen to avoid clumps in suspension. In some cases thoroughly shaking in a shaking machine will afford a satisfactory antigen, in others it must be filtered through a filter paper.



FIG. 69.—Macroscopic Agglutination of *B. cholerae suis* by Dorset-McBryde-Niles Serum. From left to right tubes show, first, complete agglutination, heavy sediment, clear supernatant liquid; in each succeeding tube the sediment becomes less, the turbidity greater, the tube at the right showing uniform cloudiness and no sediment, no agglutination. (Orig. Giltner.)

2. The antiserum may consist of immune serum—a rabbit immunized to the typhoid bacillus may be used to furnish the serum—or hog cholera serum or virus may be used with *B. typhosus* and *B. cholerae suis* respectively.

3. The following table shows the various combinations of serum, antigen and salt solution to give definite dilutions. Physiological salt solution should be used in diluting the serum.

Tube.	Antigen.	Serum Diluted 1-10.	Salt Solution.	Dilution.
	c.c.	c.c.	c.c.	
1	4	1.0	0.0	1-50
2	4	0.5	0.5	1-100
3	4	0.2	0.8	1-250
4	4	0.1	0.9	1-500
		Serum diluted 1-100.		
5	4	0.5	0.5	1-1000
6	4	0.2	0.8	1-2500
7	4	0.1	0.9	1-5000

4. Shake all tubes well and incubate at 37° C. for twenty-four hours and record the results.

Microscopic Test. 1. If this test is carried out during the same period with the macroscopic test, a small loopful of the dilution from any tube may be transferred to a clean cover-glass placed on a hanging drop slide and the edges sealed with vaselin or oil. It may then be examined with a microscope.

2. If done independently of the macroscopic test, prepare the suspension of organisms in one test tube and the dilutions of serum in others.

Mix a loopful of the diluted serum with a loopful of the antigen on a clean cover-glass, mount on a concave slide and observe with a microscope for a period of thirty minutes to one hour.

3. Give results and any conclusions in detail.

REFERENCES

- KOLMER: Infection, Immunity and Specific Therapy (1915), pp. 68; 69, 79, 152, 266-291.
 MARSHALL: Microbiology (1911), pp. 488, 567-570.
 McFARLAND: Pathogenic Bacteria and Protozoa, 7th Ed. (1912), pp. 149-152.
 GILTNER: Studies of Agglutination Reactions in Hog Cholera during the Process of Serum Production (Preliminary) Tech. Bul. 3 (1909), Mich. Agr. Expt. Sta.
 GILTNER: Same title Tech. Bul. 8 (1911), Mich. Agr. Expt. Sta.
 ZINSSER: Infection and Resistance (1914), pp. 218-247.

EXERCISE 9. A STUDY OF FILTERABLE VIRUSES

Apparatus. Physiological salt solution; Chamberland filter with water-suction or air pump and pressure gage; sterile flasks; clinical thermometer; syringe; flasks of bouillon, 50 c.c. in each; autopsy set.

Culture. Hog cholera virus (blood of hog sick with cholera).

Method. 1. Preparation of the Filter. If the filter has been used once clean it by:

- (a) First rinsing with cold water under the tap.
- (b) Force about 1 liter of cold distilled water through it.
- (c) Then a solution consisting of 1 gm. KMnO_4 and 6.5 gms. HCl in 1000 gms. water.
- (d) Next, 1000 c.c. of a solution of 1% oxalic acid.
- (e) Boiling water is then forced through the filter until the liquid which runs through is free from acid.
- (f) Lastly, cold distilled water must be forced through the filter.

Thus treated, any organic residue is destroyed and the filter is as good as new.

This method of purification must always be used *immediately after* using a filter. Filter candles *must not* be left twenty-four hours without cleaning.

A new filter may be prepared for use by forcing through it a large quantity of boiling distilled water and finally cold distilled water.

The amount of liquid necessary to force through the filter for cleaning varies with the size of the filter. The ordinary 8 inch filter should receive the full amount (1000 c.c.) of each solution and distilled water for efficient purification.

Filters are best sterilized by being set up ready to use and autoclaved. (See Fig. 71 for one method.)

2. Procure some hog cholera virus and after diluting it

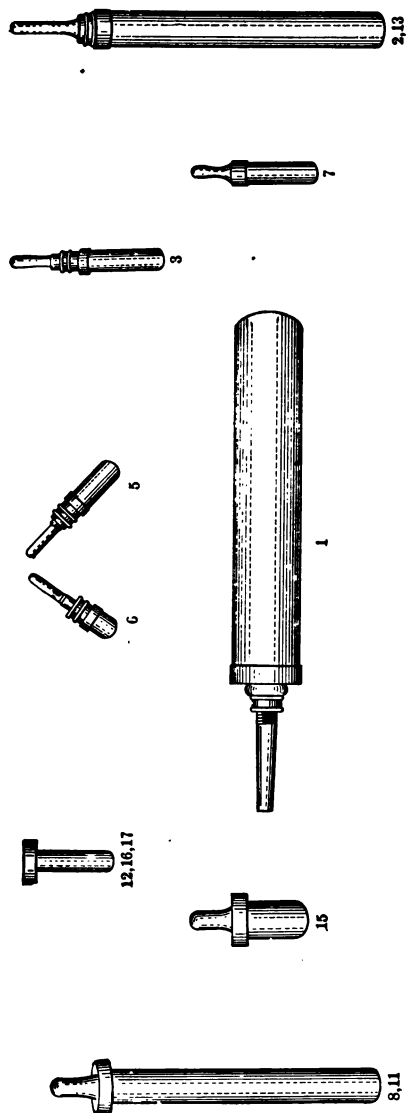


FIG. 70.—Various Types of Bacterial Filters.

with equal parts of physiological salt solution, pass it through a clean, sterile, Chamberland filter at a pressure not to exceed one atmosphere and during a time not to exceed one hour.

3. Make sub-cultures of the filtrate by introducing 1 c.c. into each of several flasks of bouillon containing 50 c.c. each. Take every precaution against contamination. Also make microscopical preparations.

4. If no growth results under 2 inject 2 c.c. into the muscles of a 50 lb. pig. Make daily observations of the pig and record the temperature each day.

5. When undoubted symptoms of hog cholera have developed, kill the pig and make a careful autopsy. Save the blood in a sterile jar.

6. Repeat the experiment, using blood procured in 4 as virus.

7. By repeated filtrations and injecting into susceptible hogs, it may be proven that a living micro-organism, incapable of producing visible growth *in vitro*, passes through the filter and develops in the body of the pig.

8. State your results and conclusions in full.

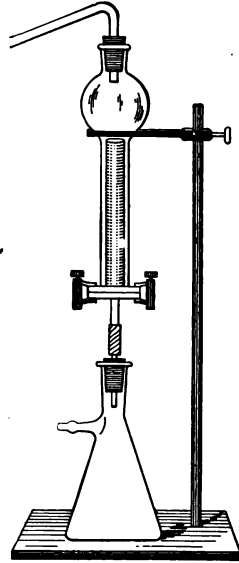


FIG. 71.—Pasteur-Chamberland Filter Adjusted for Filtration by Suction.

REFERENCES

- DORSET, MCBRYDE and NILES: Further Experiments Concerning the Production of Immunity from Hog Cholera, Bul. 102, B. A. I., U. S. Dept. Agr.
- MCBRYDE: Filtration Experiments with *B. cholerae suis*, Bul. 113, B. A. I., U. S. Dept. Agr.

GILTNER: What is the Antigen Responsible for the Production of Antibodies in Hog Cholera Serum? Tech. Bul. 13, Mich. Agr. Expt. Sta.

KOLMER: Infection, Immunity and Specific Therapy (1915), pp. 77, 78.

EXERCISE 10. THE PREPARATION OF BACTERINS OR BACTERIAL VACCINES

Apparatus. Scalpel; scissors; forceps; sterile tubes and Esmarch dishes; sterile swabs; sterile physiological salt solution; 50% alcohol; disinfectant; four agar slants; six agar tubes for plating; six sterile Petri dishes.

Culture. Infected material or specific cultures to be furnished by the instructor.

A. AUTOGENOUS BACTERINS

Method. In the preparation of an autogenous bacterin, it is first necessary to isolate the microorganism causing the disease. This is accomplished as follows:

1. If there are any unopened abscesses, open one with a sterile scalpel after first disinfecting the field with 2% compound solution of cresol and washing with 50% alcohol. Collect some of the pus on a sterile swab and suspend in sterile physiological salt solution.

2. If the abscess is already opened, using a sterile curette, obtain some of the diseased tissue at the bottom of the abscess and macerate this in sterile physiological salt solution.

3. Pour agar plates from this salt solution suspension, using at least six, plated in series of two.

4. Incubate the plates and after twenty-four hours make observations on the number and type of colonies. After forty-eight hours make transfers to agar slants of the most numerous type of colony. Colonies should be studied under low power of the microscope.

5. Grow three or four cultures of the organism on slanted

agar. Make a morphological study of the organism. After twenty-four hours wash off the growth from each tube with 3 c.c. of sterile saline solution.

6. Put the suspension all in one container, reserving 1 c.c. to be used in standardization.

Note. In the hemocytometer method for standardizing bacterins it is desirable to use a special hemocytometer with a counting chamber 0.02 mm. deep provided with a special cover-glass for counting bacteria, but if this is not accessible, an ordinary hemocytometer and cover-glass as used for blood counting may be used. If the latter, a 4 mm. objective must be used for counting.

Using the diluting pipette of the blood counting apparatus the suspension of bacteria is diluted to the desired dilution with Collison's fluid made as follows:

Hydrochloric acid, 2 cc.

Mercuric chloride 1-500, 100 cc.

Acid fuchsin, 1% aqueous solution—enough to color to a deep cherry red.

Filter before using.

The bacterial suspension is allowed to remain in the pipette eight to ten minutes to stain, then thoroughly agitated by rotating the pipette and the first few drops from the arm of the pipette discarded. The mount is then prepared and the slide placed on the stage of microscope which has been previously leveled, and the count made. The count and calculations are made as for blood counting.

7. Heat in a water bath at 60° C. for one hour. This is usually sufficient to kill the bacteria, unless they are spore producers.

8. To test the sterility of the suspension after heating, with a sterile loop make an agar streak and incubate for twenty-four hours. If growth is obtained the culture must be heated again.

B. STOCK BACTERINS

The procedure in the preparation of a stock bacterin is the same as in the preparation of an autogenous bacterin, except that the organisms used are from cultures kept in stock for that purpose.

C. POLYVALENT BACTERINS

Polyvalent bacterins are those which are prepared from several species of bacteria, e.g., *M. (Staph.) albus*, *M. (Staph.) aureus*, *Strep. pyogenes*, etc.

The suspension of each must be prepared and standard-

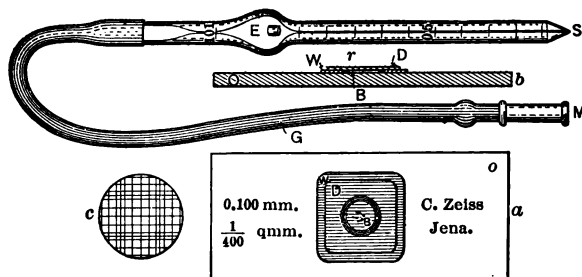


FIG. 72.—Blood-counting Apparatus for Use in Standardizing Bacterial Vaccines.

ized separately, and then the emulsions of all mixed. In this way, it is possible to have a known number of each species in the resulting product.

REFERENCES

- FITCH C. P.: A Review of the Principal Methods Used to Standardize Bacterins (Bacterial Vaccines). Report of the N. Y. State Vet. College for the year 1913-1914, pp. 207-219.
- MCCAMPBELL: Laboratory Methods for the Experimental Study of Immunity, pp. 186, 188.
- STITT: Practical Bacteriology, Blood Work and Parasitology, 2d Ed. (1911), pp. 143-145.
- KOLMER: Infection, Immunity and Specific Therapy (1915), pp. 611-661.
- ZINSSER: Infection and Resistance (1914), pp. 328-357.

EXERCISE 11. TO DEMONSTRATE OPSONINS AND TO DETERMINE THE OPSONIC INDEX

Apparatus. Several small test tubes; forty-five small mixing pipettes; sterile citrated salt solution; Wright's stain; suspension of leucocytes; normal serum; patient's serum.

Culture. Organism producing the disease.

Method. 1. The small mixing pipettes are made by drawing out 4 to 5 mm. glass tubing to a long, fine capillary tube; and providing with a small rubber bulb. (Consult the instructor for the method.)

2. Prepare the suspension of leucocytes by collecting a few cubic centimeters of blood from any animal and immediately place in three or four volumes of citrated salt solution.

Centrifuge and wash at least three times, being careful not to pipette off any of the cells during the washing process.

After the last washing, pipette off the supernatant liquid and lay the tube in as nearly a horizontal position as possible for about twenty-five to thirty minutes. At this time there will appear an upper whitish layer of cells composed almost exclusively of leucocytes.

Pipette off the leucocytes. They should be used within five to six hours from the time the blood is collected.

3. *Bacterial Suspension.* Transfer a loopful from an eighteen to twenty-four hour agar culture to 2 or 3 c.c. of physiological salt solution and mix well. The suspension must be carefully made to avoid clumps and some method of standardization used so that successive tests will be comparable. (The nephelometer may be used for this purpose—see an instructor.)

4. Collect the patient's serum and normal serum at the same time and under the same conditions in order that the results may be comparable. The blood is collected in a

small test tube and either allowed to clot and the serum removed, or it is defibrinated and centrifuged. In either case the serum should be used within three to four hours from the time the blood is drawn.

5. Make the test as follows:

a. With a diamond point or wax pencil make a mark on the drawn out arm of the mixing pipette about 2 cm. from the end.

b. With the aid of a rubber bulb on the opposite end, draw a column of the bacterial suspension up to the mark, admit a bubble of air, then draw a column of leucocytes up to the mark and another bubble of air, then a column of the serum to be tested.

c. Mix these by forcing out on a glass slide or into a small test tube and drawing up again, repeating once or twice, being careful to avoid introducing air bubbles.

d. Finally draw up the mixture into the pipette and seal the end of pipette in the flame, using care not to heat the mixture.

e. Incubate fifteen minutes with frequent shaking.

f. Then place a drop on a slide and make a thin film made as in the preparation of a blood film, dry and stain with Wright's or Jenner's stain.

6. Repeat the experiment, using the normal serum.

7. With an oil immersion lens count the number of organisms taken up by fifty leucocytes on each slide and calculate the average number taken up by each. The result is the opsonic power of the serum.

8. The *opsonic index* is the ratio of the opsonic power of the suspected serum to that of the normal serum.

Example. If the average number of bacteria taken up by the leucocytes in the presence of the suspect serum is 5.6 and the average number taken up by the leucocytes in the presence of the normal serum is 4.8, the *opsonic index* of the suspect serum is determined as follows: $5.6 \div 4.8 = 1.16 = \text{opsonic index}.$

McC Campbell's Modification of the Opsonic Test. 1. Prepare the bacterial suspension as above and add 0.8% sodium citrate.

2. (a) With a blood diluting pipette, draw the bacterial suspension up to the mark 0.5.

(b) With the same pipette draw up the same amount of blood collected from the patient, then draw both into the bulb and mix quickly.

(c) Place a flat rubber band around the ends of the pipette and incubate fifteen minutes. Prepare film, and stain.

3. Repeat the experiment, using normal blood. The opsonic index is determined as above.

Note. The sodium citrate is slightly antiopsonic but this factor is constant in both preparations, consequently the results are comparable.

4. Give results and any conclusions in detail.

REFERENCES

McC CAMPBELL: Laboratory Methods for the Experimental Study of Immunity (1909), pp. 44-70.

McFARLAND: Pathogenic Bacteria and Protozoa, 7th Ed., p. 307.

KOLMER: Infection, Immunity and Specific Therapy, pp. 187-205.

ZINSSER l.c. Exercise 10, p. 318.

EXERCISE 12. TO DEMONSTRATE THE PRECIPITIN TEST

This test is of importance in identifying the source of blood in legal cases and may also be used in the examination of various meat products for the presence of foreign meat substances.

It is based upon the fact that if an animal is injected at intervals of six to eight days for four or five times with any foreign protein its serum acquires the property of precipitating that specific protein even when in a very high dilution.

An antiserum for each specific protein to be tested for must be prepared by animal inoculation. Thus, if a test for human blood is to be made, an anti-human-blood serum must be used.

Apparatus. Syringe and needles; sterile cow's blood; sterile 500 c.c. Erlenmeyer flask containing glass beads; rabbit; disinfectant; sterile flasks, tubes and pipettes; sterile physiological salt solution.

Method. 1. Inject a rabbit intra-abdominally with 6 c.c. sterile, defibrinated cow blood. On the sixth or seventh day, repeat the injection, using 10 c.c. On the twelfth or fourteenth day give 12 c.c. and again on the eighteenth or twenty-first day give another 12 c.c.

2. On the twenty-fourth or twenty-eighth day draw a little blood from the rabbit and test it to determine its property of precipitating cow blood. If it has a high titre, the rabbit should be anesthetized and the blood drawn from the heart as explained in Exercises 1 and 7, pp. 295 and 309.

3. Place the blood in a sterile container and allow to clot. Draw the serum into small, sterile, glass bulbs holding 0.5 c.c. and seal the bulbs by heating the arm in a small flame, using care to avoid heating the serum. Serum collected in this way and placed in a cool, dark place will retain its precipitating properties for several months.

4. Dilute the blood to be tested with physiological salt solution. Several dilutions should be made, e.g., 1-200, 1-500, 1-1000, 1-10,000 and 2 c.c. of each dilution placed in small test tubes. Place six to eight drops of the antiserum in each tube. If the suspect serum is from the same species of animal as that used in immunizing the rabbit (in this case, cow serum), immediate precipitation will occur. After a few minutes' observation the tubes should be incubated at 37° C. for twenty to thirty minutes and the results noted.

5. If the blood is dried, as a blood stain on cloth, the quantity should be estimated and placed in a definite

THE PRODUCTION OF A HEMOLYTIC SERUM 323

quantity of salt solution so that the dilution may be approximated.

6. Give results and conclusions in full.

REFERENCES

- McFARLAND: Pathogenic Bacteria and Protozoa, 7th Ed., p. 146.
MARSHALL: Microbiology (1911), pp. 570-574.
NUTTALL: Blood Immunity and Relationship (1904).
KOLMER: Infection, Immunity and Specific Therapy (1915), pp. 70, 71, 292-315, 517-519, 844-846.
ZINSSER: Infection and Resistance (1914), pp. 248-271.

EXERCISE 13. THE PRODUCTION OF A HEMOLYTIC SERUM

For this work a rabbit will be immunized to washed sheep blood cells.

Apparatus. Sterile physiological salt solution; glass beads; small glass funnel; two 200 c.c. Erlenmeyer flasks; five or six sterile centrifuge tubes; sterile 5 c.c. pipette, with rubber bulb attached for draining off serum and salt solution in centrifuge tubes; sterile 14-gage $2\frac{1}{2}$ -inch hypodermic needle; sheep; rabbit.

Note. Chemically pure salt and distilled water should be used in the preparation of salt solution for this work and for the complement fixation test.

Method. 1. In one Erlenmeyer flask place eight or ten glass beads for defibrinating blood, plug and sterilize in hot air.

Provide the other one with a small, sterile, glass funnel and two layers of sterile cheese-cloth for filtering and defibrinating blood.

2. With an attendant holding the sheep, clip the wool over the area of the jugular vein and wash with 50% alcohol.

3. Place the thumb over the jugular furrow about half way between the head and shoulder and press on the vein so that it will become distended.

4. Thrust the needle through the skin anterior to the thumb, into the vein and draw about 20 to 30 c.c. of blood into the flask containing the glass beads.

5. Defibrinate by agitating three or four minutes and filter through two layers of cheese-cloth.

6. Mix the blood with approximately an equal amount of sterile physiological salt solution and centrifuge in sterile centrifuge tubes.

7. Draw off the supernatant fluid down to the corpuscles, using the sterile pipette with bulb attached.

8. Fill the tube with sterile salt solution and mix thoroughly by pouring from one tube to another several times.

9. Centrifuge again and repeat 6 and 7, at least five times.

10. Mix the washed corpuscles with an equal volume of sterile physiological salt solution, warm to body temperature and inject 14 c.c. into the peritoneal cavity of a rabbit.

11. Six or seven days later inject the rabbit intra-peritoneally with 20 c.c. of a 50% suspension of washed sheep blood cells and again on the fourteenth day with 24 c.c. of a 50% suspension.

12. About a week or ten days from the last injection draw a small quantity of blood from the rabbit, allow to clot, pipette off the serum and inactivate at a temperature of 56° C., for thirty minutes and titrate. (For method of titration, see Exercise No. 14.)

Note. Care must be exercised in the above operations to avoid contaminating the blood cells and they must be thoroughly washed and injected on the same day they are drawn.

13. State in full your results and any conclusions that may be drawn.

REFERENCES

See Exercise 14.

EXERCISE 14. TO DEMONSTRATE THE COMPLEMENT FIXATION TEST

The complement fixation test is one of the most complicated biological reactions used as a means of diagnosis in infectious diseases.

Apparatus. Guinea pig; rabbit; sheep; suspected serum (from aborting cow or other animal to be tested); small test tubes; test-tube rack; flasks; physiological salt solution; centrifuge tubes; disinfectant; syringe and needles.

Culture. *Bact. abortus* (or other organism depending on disease for which test is made).

I. TITRATION OF REAGENTS

Method. Four reagents other than the serum to be tested are required: 1, *complement*; 2, *hemolysin*; 3, *red blood cells from a sheep*; 4, *antigen*. Above components 1, 2 and 4 must be titrated before using in order to determine the amounts to be used in the tests.

1. *Complement*. This is contained in and obtained from fresh serum from a guinea pig. The complement is titrated for the purpose of determining the least amount which in the presence of a sufficient amount of hemolysin will produce complete hemolysis of a definite quantity of washed red blood cells from the sheep. This amount is spoken of as the *titre*.

2. *Hemolysin* (see Exercise 13). The source of hemolysin is inactivated serum from a rabbit that has been previously immunized to washed red blood cells from a sheep.

The selection of a rabbit and sheep is merely a matter of convenience. Any two animals of a different genus may be used. In the test for syphilis in man, human blood cells are usually used because more convenient to obtain in a number of laboratories.

TITRATION OF COMPLEMENT

Tubes.		1	2	3	4	5	6	7
Salt solution.....	(A)	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5
Hemolysin.....	(B)	0.1	0.1	0.1	0.1	0.1	0.1	0.0
Suspension of blood corpuscles.....	(C)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Complement.....	(D)	0.02	0.04	0.06	0.08	0.1	0.0	0.1
Result after one-half hour.....	(E)	—	*	+	+	+	—	—

Incubate all tubes in water bath at 37° C. for one-half hour and read.

A. 0.9% salt solution.

B. 1% dilution of inactivated immune rabbit serum in salt solution.

C. 1% suspension of washed sheep-blood cells in salt solution.

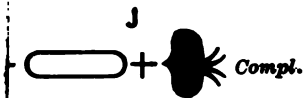
D. 20% solution of fresh guinea pig serum in salt solution (0.4 c.c. complement made up to 2 c.c. with salt solution).

E. * A variation of reaction according to strength of complement, + = complete hemolysis.

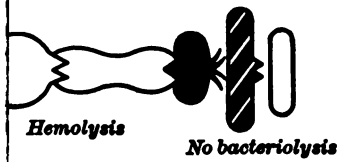
— = no hemolysis.

The inactivation is accomplished by heating to a temperature of 56° C. for one-half hour to destroy the complement. If not used for several days it is not necessary to heat, as complement is destroyed on standing. If it is to be kept for some time, preserve by adding 5% phenol sufficient to make a 0.5% solution. It is then titrated to determine the smallest quantity which will bring about a complete solution of the same quantity of washed sheep blood cells used in the titration of the complement, when in the presence of a proper quantity of complement.

3. *Antigen.* Antigen is an extract of the specific bacteria made by growing the bacteria on agar and washing off with a few cubic centimeters of salt solution, and is preserved with phenol sufficient to make 0.5% and glycerin sufficient to make 1%. The suspension is placed in a shaking machine for three hours a day for three consecutive days to obtain homogeneity.



Horse serum without
glanders ambocep. (inactivated)
bacteria (glanders antigen)



K



TITRATION OF HEMOLYSIN

Tubes.		1	2	3	4	5	6	7	8
		c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Salt solution . . .	(A)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Hemolysin	(B)	0.01	0.02	0.04	0.06	0.1	0.15	0.15	0.0
Suspension blood cells	(C)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Complement . . .	(D)	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1
Result after one-half hour	(E)	*	*	*	+	+	+	—	—

Incubate all tubes in water bath at 37° C. for one-half hour and read.

A. 0.9% salt solution.

B. 1% dilution of inactivated immune rabbit serum in salt solution.

C. 1% suspension of washed sheen-blood cells.

D. Titrated guinea pig serum diluted so that 0.1 c.c. contains 1.5 times the titre.

E. + indicates complete hemolysis.

— indicates no hemolysis.

* indicates a variation in the reaction according to the strength of the hemolysin.

The smallest quantity causing complete hemolysis is called the *titre*.

A titration of this reagent is made to determine the smallest quantity that will prevent hemolysis in the presence of 1.5 times the titre of complement and the hemolysin, sheep cells and immune serum. In other words, we must determine the smallest quantity of antigen that will fix the amount of complement used in the test.

TITRATION OF THE ANTIGEN

Tubes.		1	2	3	4	5	6	7	8	9	10	11
		c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Salt solution	(A)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Positive serum . . .	(B)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.00
Antigen	(C)	0.01	0.02	0.05	0.1	0.15	0.2	0.25	0.15	0.2	0.25	0.3
Complement	(D)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Incubate for half an hour in a water bath at 37° C., then add the hemolytic system as follows:

HEMOLYTIC SYSTEM

Tubes.		1	2	3	4	5	6	7	8	9	10	11
Hemolysin.....	(E)	c.c. 0.1	c.c. 0.1	c.c. 0.1	c.c. 0.1	c.c. 0.1	c.c. 0.1	c.c. 0.1	c.c. 0.1	c.c. 0.1	c.c. 0.1	c.c. 0.1
Suspension of blood cells.....	(F)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Results after incubation.....	(G)	*	*	—	—	—	—	—	+	+	+	+

Incubate half an hour in a water bath at 37° C. and then keep in the ice box for twelve hours and read.

A. 0.9% salt solution.

B. Inactivated serum known to contain antibodies.

C. Suspension of a culture of suspected bacteria, carbolyzed.

D. Titrated guinea pig serum diluted so that 0.1 c.c. contains 1.5 times the titre.

E. Immune rabbit serum of known titre diluted so that 0.1 c.c. contains three times the titre.

F. 1% suspension of washed sheep blood cells in salt solution.

G. * signifies a variable reaction according to the activity of the antigen.

+ signifies a complete hemolysis.

— signifies no hemolysis.

The smallest quantity of antigen (in combination with antibody) that completely fixes the complement is known as the *titre*.

II. COMPLEMENT FIXATION TEST. (Test proper)

1. *Suspected Serum*. This is drawn from the animal that is suspected of being infected with the infectious disease in question.

The blood is drawn from the jugular vein. It is allowed to clot and the serum collected. It must be inactivated before testing unless it is to be held for a week or more before applying the test, in which case inactivation is not necessary, but 1% phenol should be added as a preservative.

Note. The test proper and controls must be run *at the same time*. If several tests are run at the same time one set of controls is sufficient.

2. *Test of Suspect Serum*.

Tubes.			Test Proper.				Controls.			
			1	2	3	4	1	2	3	4
Salt solution.....	(A)	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5	c.c. 1.5
Suspect serum.....	(B)	0.1	0.02	0.04	0.04	0.0	0.0	0.0	0.0
Antigen.....	(C)	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.1
Complement.....	(D)	0.1	0.1	0.1	0.0	0.0	0.1	0.1	0.0

Incubate one-half hour at 37° C. in water bath.

3. Then add the hemolytic system.

Tubes.			Test Proper.				Controls.			
			1	2	3	4	1	2	3	4
Blood cells.....	(F)	c.c. 0.5	c.c. 0.5	c.c. 0.5	c.c. 0.5	c.c. 0.5	c.c. 0.5	c.c. 0.5	c.c. 0.5
Hemolysin.....	(F)	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1

Incubate one-half hour at 37° C. in water bath and then place in ice box for twelve hours and read.

A. Salt solution, 0.9%.

B. Suspect serum inactivated for one-half hour.

C. Antigen, two times titre.

D. Complement, 1.5 times titre.

E. 1% washed sheep blood corpuscles in salt solution.

F. Immune rabbit serum (hemolysin) diluted so that 0.1 c.c. contains three times the titre.

Control tubes 2 and 3 should show complete hemolysis. Control tubes 1 and 4 should show complete absence of hemolysis. Control tube 4 is control on the inactivation of the suspect's serum and should show absence of hemolysis.

Hemolysis in the other tubes indicates the absence of antibodies in sufficient quantity in the amount of serum used to fix complement.

4. Give all results in detail and draw conclusions.

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APPENDIX

OUTLINE FOR THE STUDY OF MICROBIOLOGY *

I. MORPHOLOGICAL AND CULTURAL MICROBIOLOGY

A. Morphology and Development.

1. Gross anatomy.

- a. Form.
- b. Size.
- c. Arrangement or grouping.
- d. Multiplication.
- e. Involution, variability and mutation.

2. Histology of cell.

- a. Wall or outer membrane.
- b. Capsule.
- c. Protoplasm, beaded forms, granules.
- d. Nuclear material.
- e. Flagella and motion.
- f. Spores.

3. Classifications and their basic features.

B. Cultural Significance.

1. Media.

- a. For morphologic and developmental studies.
- b. For cultural effects.

2. Colonies.

3. Cultural features.

4. Biochemical features.

*Adapted from Marshall, *vide* 43d Annual Report of Michigan State Board of Agriculture.

C. Staining Values.

1. Demonstrations of parts of cell.
2. Identification of species.
3. Differentiation of species.

D. Determination of Microorganisms.

1. Methods employed.
2. Differential characteristics.

II. PHYSIOLOGIC MICROBIOLOGY**A. Cell Studies.**

1. Composition of cell contents.
2. Composition of cell wall.
3. Physical products of physiological significance.
 - a. Heat.
 - b. Light.
4. Products of physiological significance of which little is known.
 - a. Pigment.
 - b. Enzymes.
 - c. Aromatic compounds.
 - d. Toxins.
5. Absorption or assimilation of foreign bodies.
6. Chemotaxis.
5. Phototaxis.
8. Aerotaxis.
9. Plasmolysis.
10. Plasmoptysis.

B. Studies in Metabolism.

1. Elements required in growth of microorganisms.
2. Respiration.
3. Nutrition.
4. Moisture.
5. Temperature of cultivation.
6. Conditions of media; reaction, composition, etc.

7. Physiologic test media.
 8. Identification and determination of species of micro-organisms by means of
 - a. Cultural physiologic methods.
 - b. Chemical tests.
 - c. Physical tests.
 - d. Biological tests.
 9. Enzymes.
- C. Studies in Association.
1. Symbiosis.
 2. Metabiosis.
 3. Antibiosis.
- D. Common Fermentative Changes Produced by Micro-organisms.
1. Studies in enzymes.
 - a. Formation.
Zymogen.
Activator.
Kinases.
 - b. Kinds.
 - c. Actions (*specificity*) and materials fermented.
 - d. Conditions under which enzymes act;
 - (1) Physical.
Temperature.
Radiation, light rays (solar, electric, etc.),
Röntgen rays, radium rays and emanations.
 - (2) Chemical and physico-chemical.
Activators; kinases, organic acids, bases,
neutral salts.
Protective agents.
Paralysors and poisons.
Concentration of solutions.
Reaction of substrate.
Extent of accumulated products.

2. Products manufactured by fermentation.

- a.* Necessary and limiting conditions of production.
- b.* Most favorable conditions of production.
- c.* Methods of determination.
 - Qualitative.
 - Quantitative.
- d.* Constancy and variability of products.
- e.* Gradation in fermentation changes.
 - Intermediate products.
 - Ultimate products.

E. Products Significant through the Intermediation of a Host.**1. Antigens.***

- a.* Cells.
- b.* Cell products.
 - Toxins, diffusible and endotoxin.
 - Bacterial proteins.
 - Enzymes.

2. Antibodies.*

- a.* Antitoxin.
- b.* Agglutinins.
- c.* Precipitins.
- d.* Opsonins.
- e.* Aggressins.
- f.* Cytolysins.
- g.* Anaphylactins.

F. Influencing Agents and Their Effects.**1. Light.**

- a.* Direct.
- b.* Diffuse.
- c.* Special.

*See p. 164, Kolmer's *Infection, Immunity and Specific Therapy* (1915).

- ### III. HYGIENIC MICROBIOLOGY

1. Man and animals.

- a. Causal agent or microorganism.
- b. History of microorganism.
- c. Vitality or persistency of microorganism.
- d. Means of dissemination and avenues of infection.
- e. Distribution of microorganism in body.
- f. Management of disease.
- g. Prevention of disease.
- h. Care of dead from communicable diseases.

B. Surgical Significance.

1. Wounds.
2. Abscesses.
3. Septicemia and pyemia.
4. Malignant growths.
5. Operations.

C. Susceptibility and Immunity.

1. Natural.
 - a. Race.
 - b. Species.
 - c. Age.
 - d. Individual idiosyncrasies.
 - e. Body components.
2. Acquired, active or passive.
 - a. Devitalization.
 - b. Hereditary predisposition.
 - c. One attack of disease.
 - d. Vaccines.
 - e. Bacterins.
 - f. Toxins.
 - g. Other bacterial products.

D. Serum Therapy—Microbial Therapeutics.

1. Diagnostic agents.
 - a. Tuberculin.
 - b. Mallein.
 - c. Bacterial suspensions.
 - d. Diphtheria toxin (Schick).
 - e. Luetin.
2. Remedial agents.
 - a. Antitoxins.
 - b. Serums.
 - c. Vaccines.
 - d. Bacterins.

E. Disinfection and Antisepsis.

1. Agents employed.
 - a. Mode of action.
2. Determination of values, phenol coefficient.
3. Methods.

F. Sanitary Studies.

1. Water analysis.
 - a. Methods.
 - b. Interpretation of results.
2. Water contamination and filtration.
3. Sewage analysis.
 - a. Methods.
 - b. Interpretation of results.
4. Sewage destruction.
 - a. Aerobic—filtration.
 - b. Anaerobic—septic tank.
 - c. End products.
5. Ventilation.
 - a. Currents as means of dissemination.
 - b. Filtration and washing of air.
 - c. Germ content of air.
 - d. Methods of analysis.
 - e. Interpretation of results of analysis.
6. Foods.
 - a. Poisonous.
 - b. Infected.

IV. DAIRY**A. Milk Supply.**

1. Communicable diseases conveyed through milk.
 - a. Kinds of microorganisms.
 - b. Avenues of transmission.
 - c. Prevention.

2. Environment of animals and conditions of milking.
 - a. Stabling.
 - b. Feeding.
 - c. Milker.
 - d. Utensils.
 3. Bacterial content of milk in udder.
 - a. Non-pathogenic microorganisms.
 - b. Pathogenic microorganisms and antibodies.
 - c. Conditions of growth in udder.
 - d. Abnormal udders.
 4. Bacterial action on constituents of milk.
 - a. Proteins.
 - b. Butter fat.
 - c. Lactose.
 - d. Mineral constituents.
 5. Analysis of air of stables.
 - a. Before cleaning.
 - b. Immediately after cleaning.
 - c. Before feeding.
 - d. Immediately after feeding.
 - e. Analysis of out-door air.
 6. Determination of value of staining.
 7. Determination of value of aeration.
 8. Determination of value of cooling.
 - a. Simple cooling.
 - b. Cooling and keeping cool.
 - c. Cooling and warming, then cooling.
 9. Cleansing of utensils.
 - a. Methods and their values.
 - b. Water analysis.
 10. Milk control.
- B. Pigment in Milk and Cheese.
1. Kinds.
 2. Character.

3. Condition of formation.
 4. Control.
- C. Fermentations in Milk, Butter and Cheese.
1. Kinds.
 - a. Lactic.
 - b. Butyric.
 - c. Alcoholic.
 - d. Gaseous.
 - e. Peptic.
 - f. Rennet.
 - g. Ropy.
 - h. Soapy.
 - i. Taints.
Bitter flavor, barn-yard, tallowy.
 - j. Special.
Kephir, koumiss, matzoon, leben, yoghurt, etc.
 - k. Natural enzymes (galactase).
 - l. Antibody formation (agglutinins, etc.).
 2. Microorganism involved.
 - a. Its life history.
 3. Nature of fermentation.
 4. Constituents acted upon.
 5. Products.
 6. Conditions influencing it.
 7. Controlled or fostered.
- D. Pasteurization and Sterilization.
1. Determination of significance of each.
 2. Methods employed.
 3. Practical utilization.
- E. Starters.
1. Natural.
 - a. Sour milk.
 - b. Sour cream.
 - c. Buttermilk.
 - d. Others.

2. Artificial.
 - a. Pure cultures.
 - b. Mixed cultures.
3. Value determined.
4. Preparation.
5. Employment.
6. Constancy.
7. Influencing conditions.
8. Facts governing amounts to employ.

F. Butter.

1. Microorganisms present.
2. Microorganisms compared with those of milk.
3. Environmental condition for bacterial life changed.
4. Quality.
 - a. Influenced by pasteurization of the cream.
 - b. Influenced by growth of microorganisms.
 - c. Factors influencing stability.
 - d. Methods of preservation.
5. Decomposition.
 - a. Products.
 - b. Factors influencing.
 - c. Correlation between the presence of certain groups of organisms and specific flavors.
6. Significance of casein and buttermilk in butter.

G. Cheese.

1. Kinds of microorganisms employed in different cheeses.
2. The study of microorganisms in the ripening process.
3. Influence of microorganisms on aroma and flavor.
4. Keeping values.

H. Preservatives.

I. Disinfectants utilized.

V. SOIL

A. The Making of Soil.

1. Microorganisms in soil.

- a.* Number at different depths and in different soils.
- b.* Kinds at different depths and in different soils.
- c.* Character of microorganisms found.
- d.* Rate of growth.

2. Disintegration of inorganic material.

3. Decomposition of organic material.

- a.* Celluloses.
- b.* Starches and sugars.
- c.* Proteins, etc.

4. Action of iron and sulphur bacteria.

B. Ammonification.

C. Nitrification—The nitroso- and nitro-processes.

1. Conditions influencing.

- a.* Physical.
- b.* Reaction.
- c.* Temperature.
- d.* Supply of oxygen.
- e.* Amount of organic matter present.
- f.* Moisture.

D. Denitrification.

1. Factors influencing the loss of nitrogen.

E. Nitrogen Fixation.

- 1. Symbiotic.
- 2. Nonsymbiotic (aerobic and anaerobic).

VI. PLANT

A. Nitrogen Accumulators.

1. Microorganism involved.
2. Cultural characteristics.
3. Formulation of nodules.
4. Character of nodules.
5. Conditions under which they form.
6. Determination of nitrogen accumulations.
7. Significance of nodules.

B. Microbial Diseases.

1. Kinds.
2. Microorganisms found as causal agents.
3. Cultural characteristics.
4. Resistance of microorganisms.
5. Persistency.
6. Methods of treatment.
7. Pathology.

C. Microbial Decomposition of Fruits, Vegetables and Other Plant Substances.

1. Nature.
2. Microorganism studies.
3. Conditions favoring.
4. Control.
5. Structural changes.

VII. FERMENTATION

A. Factors Controlling Fermentations.

1. Presence of microorganism.
2. Purity of culture.
3. Vigor of cell.
4. Character of fermentable material.
5. Air supply.

6. Reaction of medium.
7. Temperature.
8. Concentration of fermentation solutions.
9. Concentration of products of fermentation.

B. The Production of Enzymes by Microorganisms.

1. Formation of enzyme in cell.
2. Its secretion by the cell.
3. Determinative methods for study.
4. Environmental influences.

C. The Fermentations.

General.

1. The Enzymes.

a. Hydrolytic of

Carbohydrates = Carbohydrases.

Cellulases.

Hemicellulases.

Glycogenases.

Dextrinases.

Inulinase.

Saccharase.

Lactase.

Maltase.

Trehalase.

Raffinase.

Amygdalase.

Tannase.

Pectase, etc.

Fats = Esterases.

Lipases of natural fats.

Stearinases, etc.

Proteins = Proteinases.

Peptases.

Tryptases.

Ereptases, etc.

3. Products resulting.

Special.

1. Alcoholic.
 - a. Beer and distilled liquors.
 - b. Wine, cider and other fermented fruit juices.
 - c. Ginger beer.
 - d. Koumiss, etc.
2. Acetic acid.
 - a. Vinegar.
 - b. Mashies.
 - c. Foods.
3. Lactic acid.
 - a. Milk.
 - b. Mashies.
 - c. Foods, sauer kraut, brine pickles, etc.
 - d. Ensilage.
4. Butyric acid.
 - a. Milk.
 - b. Mashies.
 - c. Foods.
5. Ammoniacal.
 - a. Urea, uric and hippuric acid.
 - b. Proteins and their nitrogenous fractions.
6. Proteolytic.
 - a. Proteins, albumins.
 - b. Proteoses, albumoses.
 - c. Peptones.
 - d. Peptids.
 - e. Amino-acids.
 - f. Amins and other ammonia derivatives.
 - g. Ptomains.
 - h. Leucomains.
 - i. Non-nitrogenous organic acids.
 - j. Alcohols.
 - k. Ammonia, H_2S , and other gases.

7. Nitrification.
8. Denitrification.
9. Ammonification.

VIII. FOOD AND DRINK PRESERVATION

A. Preservation of Foods.

1. Freezing.
2. Cold storage.
3. Salting.
4. Drying, evaporating or concentrating.
5. Smoking.
6. Corning.
7. Canning.
8. Chemical preservatives or antiseptics.
9. Preserving.
10. Pressure.
11. Fermentations.

B. Preservation of Drinks.

1. Pasteurizing and sealing.
2. Cold storage.
3. Chemical preservatives.
4. Carbonating.
5. Filtration.
6. Fermentation.

CLASSIFICATION OF MIGULA (MODIFIED)

Order.	Family.	Genus.	Species.	Variety.
I. EUBACTERIA (true bacteria)	Coccaceæ round forms	<i>Streptococcus</i> . . . division in 1 plane, no fla- gella	<i>pyogenes</i> <i>erysipelatus</i>	
		<i>Micrococcus</i> . . . division in 2 planes, no fla- gella	<i>tetragenus</i> <i>pyogenes</i>	<i>aureus</i> <i>albus</i>
		<i>Sarcina</i> division in 3 planes, no fla- gella	<i>lutea</i>	
		<i>Planococcus</i> . . . division in 2 planes, flagella	<i>agilis</i>	
		<i>Planosarcina</i> . . . division in 3 planes, flagella	<i>mobilis</i>	
		<i>Bacterium</i> (straight rods) (non-flagellate)	<i>lactis acidi</i> <i>bulgaricum</i> <i>aerogenes</i> <i>abortus</i> <i>tuberculosis</i>	
	Bacteriaceæ rod forms.	<i>Bacillus</i> (straight rods) (flagellate)	<i>fluorescens lique-</i> <i>faciens</i> <i>mycoides</i> <i>prodigiosus</i> <i>typhosus</i> <i>coli</i>	
		<i>Pseudomonas</i> . . . (straight or ir- regular rods, polar flagella)	<i>radicicola</i> <i>campestris</i>	
		<i>Spirosoma</i> comma to spi- ral forms, stiff, no flagella.	<i>nasale</i>	
		<i>Microspira</i> comma- shaped, simple curve, general- ly polar fla- gella.	<i>comma</i> <i>deneke</i> <i>finkleri</i>	
	Spirillaceæ curved (comma) or spiral forms.			
Suborder.				
A. Haplobacte- rinæ.				
(lower bac- teria.)				

CLASSIFICATION OF MIGULA (MODIFIED)—*Continued*

Order.	Family.	Genus.	Species.
I.			
EUBACTERIA (true bact.)	{	<i>Spirillum</i>	{
Suborder.		cork screw, several turns, non-flexible spiral, polar flagella.	
A.		<i>Spirocheta</i>	{
<i>Haplobacterinæ</i> (lower bacteria).		flexible spirals, motile, no flagella.	
			<i>rubrum</i>
			<i>obermeieri</i>
See pp. 10 and 56-62, Marshall's Microbiology.			
I.	{	<i>Chlamydothrix</i>	{
EUBACTERIA (true bacteria).		unbranched threads, uniform in diameter.	
Suborder		<i>Crenothrix</i>	
B.	{	unbranched threads, filaments enlarged at free end.	{
<i>Trichobacterinæ</i> (higher bacteria).		<i>Phragmidiothrix</i>	
		branched and unbranched filaments. Cell division in 3 planes.	
	{	<i>Cladothrix</i>	{
		dichotomous branching, uniform diameter.	
		<i>Thiothrix</i> .	
	{	threads, non-motile and attached; sheath; gonidia.	{
		<i>Beggiatoa</i>	
		no sheath, flat cells, motile with undulating membrane; no gonidia.	
II.	{	<i>Beggiatoaceæ</i>	{
THIOBACTERIA (sulphur bacteria).		cells contain sulphur granules.	
		<i>Rhodobacteriaceæ</i>	
	{	cells contain bacteriopurpurin, sometimes sulphur granules.	{
		5 sub-families.	
		1 {	
		<i>Thiocystis</i>	{
		<i>Thiocapsa</i>	
		<i>Thiosarcina</i>	
		2 <i>Lamprocystis</i>	{
		3 <i>Thiopedia</i>	
		4 {	
		<i>Amæbobacter</i>	{
		<i>Thiothece</i>	
		<i>Thiodictyon</i>	
		5 {	{
		<i>Chromatium</i>	
		<i>Rhabdochromatium</i>	
		<i>Thiospirillum</i>	

SPECIAL MEDIA

Litmus lactose agar for demonstrating acid production of microorganisms: Prepared the same as ordinary nutrient agar (see Exercise 9, Part I), with the exception that 1% lactose and 2% of the standard azolitmin solution is added just after filtration, while the agar is still hot, and well mixed through the agar before tubing. Sterilize by Tyndall method.

Dextrose calcium-carbonate agar for showing acid formation by microorganisms: Prepared the same as ordinary nutrient agar, with the exception that 1% dextrose and 1% CaCO_3 are added to the hot agar just after filtration. The added chemicals must be mixed well through the agar and care must be taken during tubing that the CaCO_3 remains in homogeneous suspension throughout the medium. Sterilize by discontinuous method.

Sour whey for determining the acid-destroying power of microorganisms: Inoculate sweet milk with a pure active culture of *Bact. lactis acidi* or *Bact. bulgaricum* as desired, and place at about 30° C. Allow the maximum acidity to form, cut the curd and heat in flowing steam for twenty or thirty minutes. Strain through clean cheese-cloth and allow to drain. Filter through filter paper. If clear whey is desired, it will be necessary to clear the medium with egg albumin.

Butter fat for demonstrating fat decomposition: Melt butter at about 100° C. and allow the casein to settle. Decant the clear fat, place about 8 c.c. in sterile test tubes and sterilize by the intermittent method.

Other kinds of fat may be prepared similarly.

Fermented agar for making solid synthetic media and for testing food requirements and selective powers of bacteria: 1. Place a weighed amount (three parts) of agar in a large bottle and to this add 200 parts of distilled water.

2. Cover the mouth of the bottle with parchment paper

Ammonium sulphate, $(\text{NH}_4)_2\text{SO}_4$	0.40 gm.
Magnesium sulphate, MgSO_4	0.05 gm.
Dibasic acid potassium phosphate, K_2HPO_4 ..	0.10 gm.

Sodium carbonate, Na_2CO_3	0.60 gm.
Calcium chloride, CaCl_2	Trace
Distilled water.....	1000.0 c.c.

Winogradski's medium for symbiotic nitrogen-fixation:
nitrogen-free.

Dibasic acid potassium phosphate, K_2HPO_4 ..	1.00 gm.
Magnesium sulphate, MgSO_4	0.50 gm.
Sodium chloride, NaCl	0.01 gm.
Ferric sulphate, $\text{Fe}_2(\text{SO}_4)_3$	0.01 gm.
Manganese sulphate, MnSO_4	0.01 gm.
Dextrose, $\text{CH}_2\text{OH}(\text{CHOH})_4\text{CHO}$	20.00 gms.
Distilled water.....	1000.00 c.c.

Gelatin for cultivating phosphorescent halophilic organisms: Prepared as ordinary gelatin with the addition of 3% salt. The reaction is made -20° .

Fermented cider for the cultivation of acetic bacteria:
Inoculate unfermented cider with *Sacch. ellipsoideus* and allow to proceed until the evolution of gas ceases. Filter, place in tubes and flasks as desired. Pasteurize.

MEDIA FOR SOIL MICROBIOLOGY.

Soil extract: 1. Boil 1 kg. of good rich garden soil with 2 liters of tap water for two hours over the free flame.

2. Pour off the turbid liquid, mix some talc and filter through a double filter paper. If the first filtrate is turbid refilter through the same paper.

3. Make up to 800 c.c. with tap water.

4. Place in tubes or flasks as desired and autoclav.

Soil extract agar is prepared by adding 1.5% washed agar to the soil extract prepared as above.

Soil may be plated either in soil extract agar (or other special agar) or in ordinary agar, gelatin, etc. On account of the diversity of the requirements of the various species of

microorganisms in soil, no one medium will suffice for the cultivation of all species. Emphasis is therefore not laid on any particular medium for plating soils.

Omeliansky's medium for anaerobic cellulose fermentation:

Filter paper (in strips). Cotton, straw, or starch may be substituted for filter paper.	2.0 gms.
CaCO ₃	20.0 gms.
K ₂ HPO ₄	1.0 gm.
MgSO ₄	0.5 gm.
(NH ₄) ₂ SO ₄	1.0 gm.
NaCl.....	Trace
Distilled water.....	1000.0 c.c.

Method. 1. Introduce substances in order named into 1000 c.c. distilled water.

2. Stir to dissolve all soluble substances and tube while insoluble substances are in homogeneous suspension, placing about 10 c.c. in each tube.

3. Sterilize in autoclav.

Media for studying urea decomposition: Urea broth, gelatin and agar are generally prepared by adding 1% to 2% urea to the ordinary media. This medium favors the growth of *B. coli*, *B. proteus*, *B. erythrogenes*, etc.

Ordinary media to which 10% urea has been added favors the growth of *B. pasteurii*, a spore-producing bacterium.

Urea gelatin and agar may be prepared by adding 1 c.c. of a 15% aqueous solution of urea to each tube of the ordinary media after sterilization, and then heating the tubes again. This is the method preferred because the addition of urea reduces the solidifying power of the gelatin. A small amount of urea is converted into ammonia by heating in the steam, but this has little influence on the results obtained in the experiment. Heating in the autoclav is to be avoided!

Albuminoid-free culture solutions for studying urea decomposition:

I. Soil extract.....	100 c.c.
K ₂ HPO ₄	0.05 gm.
Urea.....	5.00 gms.

II. *Sohnsen's solution.*

Tap water.....	100.00 c.c.
Urea.....	5.00 gms.
K ₂ HPO ₄	0.05 gm.
Ammonium or calcium malate, or, calcium citrate or tartrate.	0.50 to 1.00 gm.

B. pasteurii will not grow in these solutions as it requires the presence of albuminoids in the medium.

Solutions for cultivating nitrifying bacteria:

I. Distilled water.....	1000.0 c.c.
(NH ₄) ₂ SO ₄	1.0 gm.
K ₂ HPO ₄	1.0 gm.
MgSO ₄	0.5 gm.
NaCl.....	2.0 gms.
FeSO ₄	0.4 gm.

Add basic MgCO₃ after sterilizing.

This solution is adapted for relatively increasing the nitrite bacteria.

II. Distilled water.....	1000.0 c.c.
NaNO ₂	1.0 gm.
K ₂ HPO ₄	0.5 gm.
MgSO ₄	0.3 gm.
NaCl.....	0.5 gm.
Na ₂ CO ₃	0.3 gm.

This solution causes a greater relative increase in the nitrate producers.

III. The same as solution I, but instead of MgCO_3 CaCO_3 is added after sterilizing. This solution stimulates the simultaneous growth of both organisms, as in nature.

Culture solutions for denitrification studies. Nitrate broth or agar. Add 1 c.c. of a 1% solution of sodium or potassium nitrate to tubes of ordinary broth or agar (melted), mix well and re-sterilize.

Giltay's solution.

KH_2PO_4	2.0 gms.
MgSO_4	2.0 gms.
KNO_3	1.0 gm.
CaCl_2	0.2 gm.
Fe_2Cl_6 solution.....	2.0 drops
Citric acid.....	5.0 gms.

Method. 1. Dissolve the above substances in 800 c.c. of distilled water (solution I).

2. Add a few drops of phenolphthalein and, using a pipette, drop in just enough 10% NaOH to turn the solution a faint pink.

3. Dissolve 10 gms. dextrose in 200 c.c. of distilled water (solution II).

4. Mix solutions I and II very thoroughly.

5. Sterilize in the autoclav at 15 lbs. pressure for ten minutes. (Lipman and Brown.)

Giltay's agar is prepared by adding 1.5% washed agar to the above solution. Boil until dissolved. Filter through absorbent cotton. Sterilize in autoclav.

Mannit solution for nitrogen-fixing organisms.

Mannit.....	15.0 gms.
K_2HPO_4	0.2 gm.
MgSO_4	0.2 gm.
NaCl	0.2 gm.
CaSO_4	0.1 gm.
CaCO_3	5.0 gms.
10% FeCl_6 solution.....	1.0 drop

Method. 1. Add the above chemicals to 1000 c.c. distilled water.

2. Titrate using phenolphthalein and neutralize using normal NaOH.

Do not filter. The presence of CaCO_3 offers an additional means of isolating *Azotobacter*, as these organisms are found in soil in much greater numbers around the particles of calcium carbonate.

3. Sterilize at 120°C . (autoclav), for ten minutes.

Mannit agar is prepared by adding 1.5% washed agar to the above solution, boiling until the agar is wholly dissolved and sterilizing as above. *Do not filter.*

Nitrogen-free ash agar for cultivation of Ps. radicola.

1. Stir 5 gms. of wood ashes (beech, elm, maple) into 1000 c.c. distilled water for two to three minutes *only*. Filter.

2. Add 1% washed agar.

3. Heat in steam for thirty minutes.

4. Then add 1% commercial saccharose.

5. Boil five minutes over a free flame.

6. Strain while hot through several thicknesses of clean cheese-cloth. This may be filtered if desired.

7. For Exercise 9, Soil Microbiology, tube, placing about 6 cm. of agar in the large test tubes with foot, the rest in ordinary test tubes. Sterilize. (Tyndall method.)

Nitrogen-free solution may be prepared as above, omitting the agar.

Congo-red agar for differentiating Ps. radicola from Bact. tumefaciens:

Distilled water	1000.00 c.c.
Saccharose	10.0 gms.
K_2HPO_4	1.0 gm.
MgSO_4	0.2 gm.
Washed agar	15.0 gms.
Congo-red	0.1 gm.

Solution for sulphate reduction:

Tap water	1000.0 c.c.
K ₂ HPO ₄	0.5 gm.
Sodium lactate	5.0 gms.
Asparagin	1.0 gm.
MgSO ₄	1.0 gm.

A few drops of FeSO₄ solution. Sterilize in the autoclav.

WATER ANALYSIS MEDIA

Culture media for standard bacteriological water analysis must contain ingredients of a special nature.

Ingredients. 1. Distilled water in place of tap water.

2. Infusion of fresh lean meat instead of meat extract.

3. Witte's peptone (dry, from meat).

4. No salt.

5. Gelatin of the best French brand and as free as possible from acids and other impurities.

6. Commercial agar of as high a grade of purity as possible. Agar may be purified by washing.

7. Dextrose, lactose, saccharose, etc., of sugar media, chemically pure.

8. A 1% aqueous solution of Kahlbaum's azolitmin may be used in place of litmus.

Sterilization. Sterilize media in the autoclav at 120° C. (15 lbs. pressure) for fifteen minutes. A shorter period than this may result in incomplete sterilization, a longer period will probably result in inversion and caramelization of the sugars and in lowering the melting-point of the gelatin. *Have the sterilizer hot when the medium is inserted so that heating to the point of sterilization will be accomplished as quickly as possible; cool rapidly upon removing from the autoclav.*

The Tyndall (intermittent) method may be employed, heating for thirty minutes on three successive days.

Reaction. Phenolphthalein is used as indicator.

Titrate media while hot with N/20 NaOH and adjust the reaction if necessary. All media should have a $+10^{\circ}$ reaction Fuller's scale unless otherwise stated in the directions.

Distribution of Work. It may be desirable to have students work in groups in preparing media. The following plan has worked satisfactorily:

Students may work in groups of five, one of the groups preparing a sufficient quantity of medium for himself and the other four members of the group, dividing the work up as follows:

One student prepare agar shakes and litmus milk.

One student prepare gelatin.

One student prepare litmus lactose agar.

One student prepare litmus lactose bile.

One student prepare Dunham's solution and nitrate peptone solution.

In this arrangement each student must furnish the respective sterile glassware sufficient for containing the various necessary media, to the student preparing each medium.

Each student of the group must so plan his work that the medium he prepares will be finished, sterilized and ready for use at the same time as those of the remaining members of his group.

Media. *Litmus lactose agar shake.*

I. 2% washed agar.

2% peptone.

2% lactose.

2% azolitmin.

500 c.c. meat infusion.

500 c.c. distilled water.

Method. 1. Strain the meat infusion through a piece of clean cheese-cloth.

2. Place the washed agar in the distilled water, weigh,

digest over a free flame, weigh again and make up any loss with distilled water.

3. To the hot agar add the peptone and lactose and mix until dissolved; then add the strained meat infusion.

4. Titrate and adjust the reaction to 0° .

5. Add the azolitmin, boil up over the free flame and place about 100 c.c. in sterile 250 c.c. Florence flasks.

Each student will need four litmus lactose agar shakes.

II. *Litmus lactose agar.* (To be used in tubes for plating only.)

1.5% agar.

1.0% peptone.

1.0% lactose.

2.0% azolitmin solution.

500 c.c. meat infusion.

500 c.c. distilled water.

Method. 1. This agar is prepared as ordinary nutrient agar making the reaction $+10^{\circ}$, *adding the lactose and azolitmin just before tubing.*

2. Tube and sterilize by the Tyndall method.

Each student will need at least forty tubes of litmus lactose agar.

III. *Gelatin.*

15% gelatin.

1% peptone.

500 c.c. meat infusion.

500 c.c. distilled water.

Method. Prepare, tube and sterilize as for ordinary gelatin.

Salt is omitted. Reaction $+10^{\circ}$.

Each student will need forty or fifty tubes of salt-free gelatin.

IV. *Sugar-free broths and sugar broths.* (Neutral red dextrose broth.)

Method. 1. Heavily inoculate a tube of sterile broth with *B. coli* and incubate at 37° C.

2. Soak 1 lb. finely chopped lean beef in 1000 c.c. distilled water over night (twenty-four hours).

3. Strain out the meat juice and make up to 1000 c.c. with distilled water.

4. Pour the entire contents of the twenty-four-hour broth culture of *B. coli* into the meat juice and

5. Incubate at 37° C. for twelve to sixteen hours, *not longer*. *B. coli* uses the fermentable substances, inosite (muscle sugar), dextrose, etc., as food, leaving the meat juice free from fermentable substances. *If this action is allowed to proceed too long, poisonous decomposition products of the proteins are formed which will inhibit the growth of other microorganisms.*

6. Mix the peptone (1%) into a thin paste with as little water as possible and add to the twelve or sixteen-hour culture of *B. coli* in the meat juice.

7. Heat in the autoclav for twenty minutes or in the steam for one hour.

8. Titrate and make neutral to phenolphthalein.

9. Boil over a free flame for three to five minutes.

10. Add 1% dextrose and 10 c.c. of 0.5% solution of neutral red and stir until sugar is dissolved.

11. Filter until clear.

12. *Fill ten fermentation tubes for each student.*

13. Sterilize in autoclav or in flowing steam.

14. Other sugar broths are prepared by adding instead of dextrose, 1% of the sugar desired.

Practically all sugar-fermenting organisms will ferment monosaccharides such as dextrose; comparatively few will ferment the disaccharides lactose, saccharose, etc. *B. coli* will ferment all three sugars to a greater or less extent. Bacteria of the typhoid group ferment *none* of the three and those belonging to the paratyphoid group ferment dextrose but *not* lactose, therefore the use of lactose in culture

media will inhibit to a great extent the growth of the last two groups and favor the development of the organisms of the *B. coli* group. This group is by far the largest, occurs most often and in greatest numbers in sewage and like material, therefore tests for this group are used as indication of the presence of intestinal organisms in the material (water in this case) to be examined.

V. Litmus lactose bile salt medium.

Bile salts are invaluable for certain media used for water analysis as they inhibit organisms of practically all but the intestinal type.

- 20 gms. peptone.
- 5 gms. sodium taurocholate.
- 10 gms. lactose.
- 20 c.c. 2% azolitmin solution.
- 1000 c.c. distilled water.

Method. 1. Dissolve the bile salt and peptone in the water and boil.

2. Add the lactose and sufficient azolitmin to give a distinct purple tint.

3. Filter, fill into fermentation tubes and sterilize by intermittent method.

Each student needs four litmus lactose bile salt fermentation tubes.

VI. Esculin bile solution for B. coli test.

- 10.0 gms. peptone.
- 5.0 gms. sodium taurocholate.
- 0.1 gm. esculin.
- 0.5 gms. soluble iron citrate.
- 1000.0 c.c. distilled water.

Method. 1. Dissolve the ingredients in the order given, clear with egg albumen, tube and sterilize (see Prescott and Winslow's Elements of Water Bacteriology, 3d Ed., p. 279). This solution has a blue fluorescence.

VII. *Dunham's solution*; twenty-five tubes for each student. (See p. 43.)

VIII. *Nitrate peptone solution*; twenty-five tubes for each student. (See p. 44.)

IX. *Litmus milk*; twenty-five tubes for each student. (See p. 25.)

EXPLANATION OF TABLE ON PAGES 362-363

Method. *B. coli*- and *B. cholerae suis*-like organisms: Place 5 c.c. of suspected water in each dextrose and liver broth fermentation tube and 50 to 100 c.c. in a litmus lactose agar flask. Incubate at 37° C. If gas appears in time of three days, make plating on Conrad-Drigalski's agar * from one showing most of gas production. Isolate different colonies on agar slants. From the growth on the agar slants inoculate different media to subgroup the organisms and consequently to identify them.

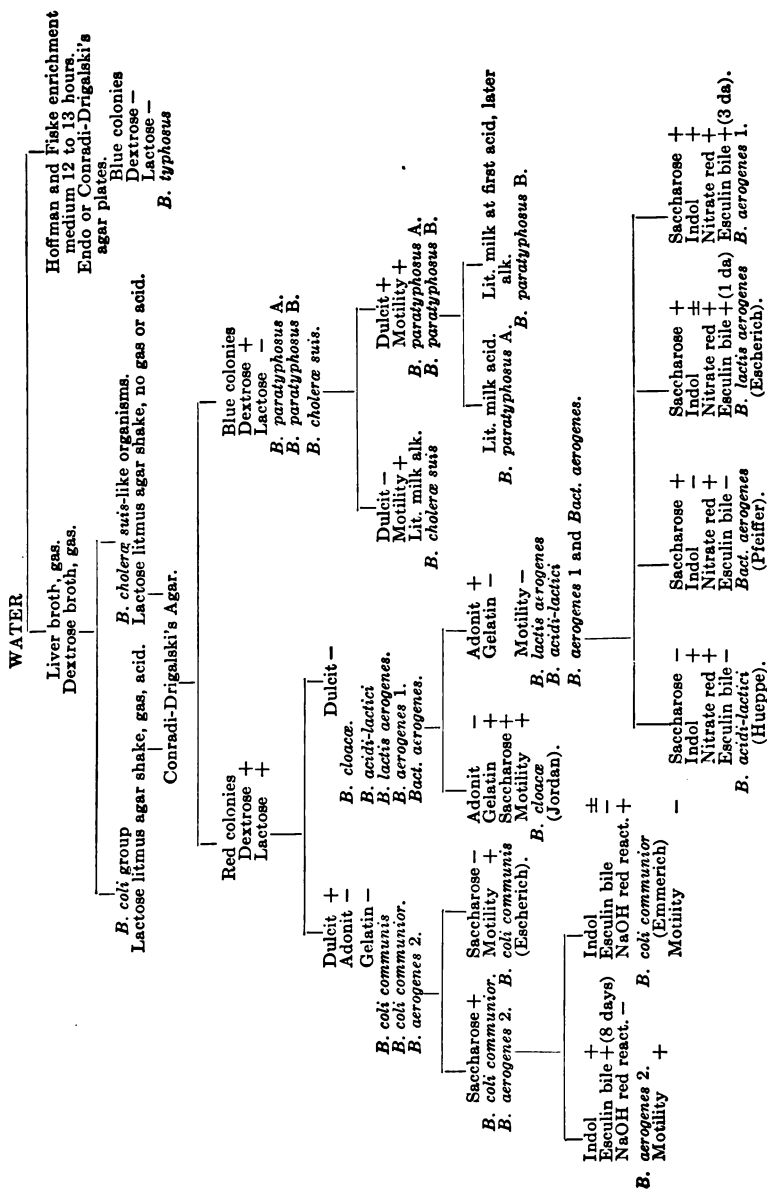
B. typhosus: Hoffman and Fiske enrichment medium. Add to the suspected water 1.0% of nutrose; 0.5% of caffein; 0.001% of crystal violet. Incubate at 37° C. for *not more than* twelve to thirteen hours. Make Endo or Conradi-Drigalski agar plates. Isolate bluish colonies, transferring to agar slant, and identify. The Widal reaction should be used for the confirmatory test.

(Data on pages 362-363 collected by O. M. Gruzit.)

* Other media for bacteriological water analyses will be found in the 1915 edition of the "Standard Methods for the Examination of Water and Sewage" published by the American Public Health Association, pp. 124-137.

This publication is the standard work; references to special phases will be found in the bibliography following each chapter.

IDENTIFICATION OF BACTERIA IN POLLUTED WATER



CHARACTERS OF *B. COLI* - *B. TYPHOSUS* GROUPS

Names of Organisms.	Dextrose.	H ₂ : CO ₂	Lactose.	Saccharose.	Dulcit.	Adonit.	Liver broth.	Inulin.	Indol.	Nitrites.	Litmus milk.	Neutral red.	Gelatin liquefaction.	Esculin bile.	Endo medium.	Conradi-Dragalek's medium.	Voges-Proskauer's reaction.	Gram's stain.	Motility.	Morphology.	Litmus lactose agar.	NaOH red reaction
<i>B. coli communis</i> (Escherich).	+	1+	+	+	+	+	+	+	+	+	+	+	+	4 dys.	+	red	+	+	+	rod	+	+
<i>B. coli communis</i> (Emmerich)	+	1+	+	+	+	+	+	+	+	+	+	+	+	+	+	red	+	...	+	rod	+	+
<i>B. aerogenes</i> 1.	+	...	+	+	+	...	+	+	+	+	+	+	+	3 dys.	+	red	+	rod	+	+
<i>B. aerogenes</i> 2.	+	...	+	+	+	...	+	+	+	+	+	+	+	8 dys.	+	red	+	rod	+	+
<i>B. acidi lactici</i> (Hueppe)....	+	1+	+	+	+	+	+	+	+	+	+	+	+	+	+	red	+	...	+	rod	+	+
<i>B. lactis aerogenes</i> (Escherich).	+	1+	+	+	+	+	+	+	+	+	+	+	+	1 dy.	+	red	+	...	+	rod	+	+
<i>Bact. aerogenes</i> (Pfeiffer)....	+	1+	+	+	+	+	+	+	+	+	+	+	+	+	+	red	+	...	+	rod	+	+
<i>B. cloacae</i> (Jordan).....	+	1+	+	+	+	+	+	+	+	+	+	+	+	5 dys.	+	red	+	...	+	rod	+	+
<i>B. cholerae suis</i>	+	1+	+	+	+	...	+	...	+	+	alk	red	+	...	+	blue	+	...	+	rod	+	+
<i>B. paratyphosus</i> A.....	+	1+	+	+	+	...	+	...	+	+	+	...	+	...	+	blue	+	...	+	rod	+	+
<i>B. paratyphosus</i> B.....	+	1+	+	+	+	...	+	...	+	+	alk	...	+	...	+	blue	+	...	+	rod	+	+
<i>B. typhosus</i>	+	...	+	+	+	+	+	+	+	+	+	+	+	+	+	blue	+	+	+	rod	+	+

+ = positive; - = negative. ± = varieties of one species variable.

COMMON DISINFECTANTS

Mercuric chloride: (HgCl_2) White crystals.

Synonyms: mercury bichloride, corrosive sublimate, bichloride of mercury.

The stock solution (40% HgCl_2 in HCl) is prepared by mixing 1 part mercuric chloride with 2.5 parts commercial hydrochloric acid. This dissolves readily and aqueous solutions of any desired dilution may be made from it much more quickly than by the use of the salt.

The pure salt is soluble in 16 parts of cold water and 3 parts of boiling water.

Mercuric chloride 1 : 1000, the solution commonly used in the laboratory for disinfecting purposes, is prepared by adding to 2.5 c.c. of the stock solution, sufficient distilled water to make 1000 c.c. of solution.

As a germicide, mercuric chloride acts in solution by combining chemically with the protein of the microorganisms. Therefore its efficiency varies in inverse proportion to the amount of dead organic matter present.

Mercuric chloride is exceedingly corrosive as is also the acid in which it is originally dissolved; therefore it should not be placed in metal containers or agateware pails, cups, etc., if the enamel is chipped sufficiently to expose the metal.

Remember that mercuric chloride is a **DEADLY POISON!** Great care must be exercised in properly labelling all bottles, etc., containing it.

Phenol: ($\text{C}_6\text{H}_5\text{OH}$) long colorless crystals that become pink upon exposure to light and air.

Synonyms: *Carbolic acid*, phenic or phenylic acid, phenyl hydrate, hydroxybenzene (or -ol).

The stock solution (95% phenol) is prepared by adding 1 part of water to 19 parts (by weight) of crystalline phenol. Solution may be hastened by placing the vessel containing the crystals in a dish of warm water.

Note. When making up the stock solution or dilutions from the stock solution always have a bottle of ethyl alcohol at hand as a remedy for burns caused by phenol. 5% phenol is prepared by adding one part of 95% phenol to nineteen parts of distilled water.

Its value as a disinfectant is increased by the fact that it acts in the presence of albuminous substances. It does not corrode metals or destroy fabrics in a 5% solution.

Liquor cresolis compositus, U. S. P.

Cresol.....	500 gms.
Linseed oil.....	350 gms.
Potassium hydroxide.....	80 gms.
Water, a sufficient quantity to make.....	1000 gms.

Dissolve the potassium hydroxide in 50 gms. of water in a tared dish, add the linseed oil, and mix thoroughly. Then add the cresol and stir until a clear solution is produced. Finally add sufficient water to make the finished product weigh 1000 gms., or more briefly: mix equal parts by weight of cresol and linseed oil-potash soap (*Sapo mollis*, U. S. P.).

This mixture is a thick, dark, amber-colored fluid which mixes readily with water in all proportions to form a clear, soapy solution. A 3% or 4% solution will accomplish the same results as 5% phenol. It is not interfered with by albuminous substances and does not destroy metals or fabrics.

Tincture of iodin, U. S. P.

Iodin.....	70 gms.
Potassium iodid.....	50 gms.
Alcohol, sufficient to make.....	1000 c.c.

Triturate the iodine and potassium iodide in a mortar to a coarse powder and transfer at once to a graduated flask. Rinse the mortar with several successive portions

of alcohol and pour the rinsings in the bottle; then add alcohol, shaking occasionally until the iodine and potassium iodide are all dissolved and the finished tincture measures 1000 c.c.

SOLUTIONS FOR CLEANING GLASSWARE

Chromic acid cleaning solution for cleaning glassware:

Potassium or sodium dichromate.....	60 gms.
Commercial sulphuric acid.....	60 c.c.
Water.....	1000 c.c.

Prepare in a flask resistant to heat, never in a heavy glass jar.

Add the potassium dichromate to about 500 c.c. water; shake well and add the sulphuric acid gradually, continually shaking with a rotary motion. The remaining water may then be added. The potassium dichromate should be all dissolved before using the solution.

This solution may be used repeatedly until oxidized to a dark green color. Heat will hasten its action.

Chromic acid cleaning solution is especially valuable for removing traces of oxidizable organic matter and neutralizing any free alkali adhering to glassware. However, effort should be made to previously remove as much extraneous matter as possible with water and a suitable brush before treating with this solution. This will economize time.

Caution. This solution contains sufficient sulphuric acid to destroy fabrics, bristles of brushes, and corrodes metal quickly. For this reason neither cloth nor brushes should be used as an immediate aid to this cleaning agent, *nor should this solution be placed in agateware utensils if the enamel is chipped, exposing the metal.*

If this solution is used cold, leave the glassware containing it, over night *on top* of desk, *never* inside of desk.

Sodium hydroxide solution for cleaning glassware and absorbing CO_2 :

Sodium hydroxide, sticks 100 gms.
Water 1000 c.c.

Use only once if the glassware is very dirty.

This solution is invaluable for cleaning greasy flasks, pipettes, etc.

Caution. This solution should not be left in contact with any glassware longer than thirty minutes as it etches the glass.

A sodium hydroxide solution of this strength is very corrosive, attacking cloth, laboratory desk tops, etc., and, therefore, should be wiped up immediately if spilled.

This strength may also be employed to absorb CO_2 in fermentation tube cultures of gas-producing organisms.

STANDARD SOLUTIONS

A. Preparation of N/10 Na_2CO_3 from titration against which normal acid is prepared.

1. Dry finely powdered chemically pure Na_2CO_3 in a drying oven at 105°C . for two hours.

2. Weigh out carefully and as accurately as possible 5.3 gms. of the dried salt.

3. Dissolve in distilled water which has been boiled previously to expel CO_2 and then cooled.

4. Make up solution to one liter, using a calibrated volumetric flask and observing temperature for which the flask was calibrated.

5. Keep this N/10 solution of Na_2CO_3 in a stoppered bottle. It should be used as soon as possible after preparation, as the Na_2CO_3 acts upon the glass and thus deteriorates.

B. Preparation of N/1, N/10 and N/20 HCl.

1. Measure out 77.5 c.c. HCl (sp.gr. 1.20) or 138 c.c.

HCl (sp.gr. 1.12) and make up to one liter with distilled water. This makes a solution just a little stronger than normal.

2. To determine its exact strength, titrate 5 c.c. with N/10 Na_2CO_3 , using phenolphthalein as the indicator.

3. Run check determinations, which should check within one- or two-tenths of a cubic centimeter.

4. From results, calculate by proportion how much a liter of the solution should be diluted to make it N/1. e.g.:

5 c.c. HCl was neutralized by 55 c.c. N/10 Na_2CO_3

\therefore HCl is N/1.1

By proportion:

$$\text{N/1} : \text{N/1.1} :: 1000 : x$$

$$x = 1100$$

Hence each liter of the HCl solution should be diluted to 1100 c.c. with distilled water to make a N/1 solution of HCl.

5. N/10 and N/20 solutions of HCl can be made by making the proper dilutions. *Always use calibrated flasks and burettes when making these dilutions.*

C. Preparation of N/1 and N/20 NaOH.

1. Weigh out roughly 41 gms. of chemically pure NaOH.

2. Dissolve in distilled water, which has been boiled to expel CO_2 and then cooled.

3. Make up to one liter, using a calibrated volumetric flask and observing the temperature for which it was calibrated. This makes a solution a little stronger than normal.

4. Determine its exact strength by titration with N/10 HCl.

5. Proceed from this point as in the preparation of N/1 HCl.

6. N/10 and N/20 solutions can be made from the N/1 solution as in the preparation of N/10 and N/20 HCl.

INDICATORS

Phenolphthalein, indicator for titration:

Phenolphthalein.....	0.5 gm.
50% alcohol (neutral).....	100.0 c.c.

A drop of a weak solution of alkali should produce permanent pink color when added to a small amount of this solution. Phenolphthalein is colorless in the presence of acid.

Kahlbaum's azolitmin solution: Dissolve 2.5 gms. of Kahlbaum's azolitmin in 100 c.c. distilled water by heating in steam for half an hour. Filter (this will filter much more readily if allowed to settle for some time; decant upon the filter). Sterilize by heating fifteen minutes each day on three successive days. Sterilization is necessary, otherwise molds and other microorganisms will grow on the organic material present, often changing the reaction.

A solution of litmus or azolitmin is often added to sugar and other media before sterilization for the purpose of detecting microorganisms which produce a change in the reaction of the media.

Litmus is a mixture of dyes obtained from the lichens *Roccella* and *Lecanora* by allowing them to ferment after the addition of ammonia and potassium carbonate. When the mass has assumed a deep blue color, the liquid is pressed out, absorbed by chalk or gypsum, and dried.

Merck's purified litmus, often used in bacteriological work, is made from commercial litmus solution by freeing it from the red pigment orcin, and drying without absorbing it by means of chalk or gypsum.

Azolitmin is a purified pigment from litmus.

SALT SOLUTIONS

Physiological salt solutions for immunity work, dilution flasks, etc.:

Sodium chloride, c.p.	8.5 gms.
Distilled water.	1000.0 c.c.

Chemically pure sodium chloride must be used for immunity work, especially for animal injection. For dilution flasks the best grade of cooking salt serves the purpose. *Salt prepared for table use cannot be used on account of its starch content.*

Normal salt solution for dilution purposes, etc., *not for immunity work:*

Sodium chloride, best commercial grade.	60 gms.
Distilled water.	1000 c.c.

Citrated salt solution for used in demonstrating opsonins:

Sodium chloride, c.p.	8.5 gms.
Sodium citrate.	15.0 gms.
Distilled water.	1000.0 c.c.

TEST SOLUTIONS

Ehrlich's test solution for indol production:

Solution I.

Para-dimethyl-amido-benzaldehyde	4 gms.
96% alcohol.	380 c.c.
HCl, conc.	80 c.c.

Solution II. Saturated watery solution of potassium persulphate (oxidizing agent).

See Exercise 44, Part I, for the method of the test.

Nitrate test solutions:**I. Phenolsulphonic acid.**

1. Mix 3 gms. of pure crystallized phenol with 37 gms. of c.p. concentrated sulphuric acid (20.1 c.c., sp.gr. 1.84) in a round-bottom flask.

2. Heat for six hours in a water bath at 100° C., keeping the flask submerged the whole time.

This may crystallize on cooling, but it can be brought into solution easily by heat.

Directions for making this test will be noted in Exercise 45, Part I.

II. Diphenylamin. A solution of 2% diphenylamin in sulphuric acid when added to a liquid containing nitrates or nitrites gives a blue color.

Diphenylamin	2 gms.
Sulphuric acid, c.p. conc.	100 c.c.

Nitrite test solutions:

Solution I. 8.0 gms. sulphanilic acid dissolved in 1000 c.c. of 5N acetic acid (sp.gr. 1.041).

Solution II. 5.0 gms. *α*-naphthylamin dissolved in 1000 c.c. of 5N acetic acid. These solutions should be kept separate and mixed in equal parts just before use.

Nessler's solution, for free ammonia:

1. Dissolve 62.5 gms. of potassium iodid in 250 c.c. of distilled water. Reserve about 10 c.c. of this solution.

2. Add gradually to the main portion a cold saturated solution of mercuric chloride, stirring constantly and increasing the quantity of mercuric chloride until a bright, permanent precipitate is formed.

3. Now add the reserved potassium iodid solution and again add the saturated mercuric chloride solution, cautiously and with constant stirring until a distinct though slight red precipitate remains.

4. Dissolve 150 gms. of caustic potash in 150 c.c. dis-

tilled water, allow the solution to cool and add it to the above solution.

5. Dilute to one liter with distilled water.
6. Allow to stand for one week and decant for use.

MOUNTING MEDIA

Canada balsam for making permanent mounts of microscopic preparations:

Canada balsam, dry, hard, for microscopic use. 4 parts
Xylol. 3 parts

This gives a mounting medium of about the right consistency. It should not "thread" when a drop is taken out with the glass rod. Balsam should be kept in a bottle stoppered with a glass bell-stopper, and having a rim arranged so that the excess of balsam taken upon the glass rod can be drained off.

Immersion oil for oil immersion objectives.

It is necessary that the immersion oil have practically the same index of refraction as glass in order to avoid dispersion of any of the light rays. Cedar wood oil having a refractive index of 1.515 to 1.520 is the usual medium interposed between the specimen and the oil immersion objective as it has approximately the same index of refraction as crown glass, 1.518. The refractive index of air is 1.000.

Chinese ink:

Burri's "Pelikan" Chinese ink. 1 part.
Distilled water. 7 parts.

Tube, using 8 to 10 c.c. per tube, sterilize in the autoclav and allow to stand two or three weeks without disturbing, for sedimentation to take place. It is to be used without shaking or disturbing any more than necessary.

STAINS

Methylen blue for differentiating living from dead yeast cells:

Methylen blue..... 0.1 gm.
Distilled water..... 1000.0 c.c.

Aqueous-alcoholic stains, fuchsin, methylen blue and gentian violet:

1. A saturated alcoholic solution of a stain is prepared by shaking frequently about 10 gms. of the stain with 100 c.c. of absolute alcohol. If the stain dissolves quickly, add more dry stain. The alcoholic solution should be slightly supersaturated.

2. Allow the undissolved stain to settle over night.

3. Decant.

4. Dilute 1 part of the alcoholic solution with 9 parts of distilled water.

Note 1. If 95% alcohol is used instead of absolute alcohol to dissolve the stain, the dilution should be made 1 : 7.

Note 2. These aqueous solutions may not keep longer than about a month, while the saturated alcoholic solutions keep indefinitely.

Note 3. The vegetative forms of bacteria stain more or less readily with all aqueous-alcoholic stains but *not with saturated alcoholic stains*. Acid-fast bacteria, e.g., *Bact. tuberculosis*, are the exception to the former.

Anilin-water gentian violet:

1. Shake 5 c.c. of anilin oil vigorously with 100 c.c. of distilled water in a stoppered bottle for several minutes.

2. Filter through a wet filter immediately before use.

3. Add 1 part of saturated alcoholic solution of gentian violet to 9 parts of the freshly prepared anilin-water and filter immediately before use.

Note. Anilin-water stains do not keep longer than about a week. The stock solutions will keep indefinitely if kept separate.

Ziehl-Nielson's carbol-fuchsin.*Solution A.*

Basic fuchsin.....	1 gm.
Absolute alcohol.....	10 c.c.

Solution B.

Carbolic acid.....	5 gms.
Distilled water.....	100 c.c.

1. Dissolve the fuchsin in the absolute alcohol. (*Solution A.*)

2. Dissolve the carbolic acid in the distilled water (*Solution B.*)

Note. *Solutions A and B* will keep indefinitely if kept separate.

3. Mix in the proportion of 10 c.c. of *solution A* to 100 c.c. of *solution B.*

Note. If *A* and *B* do not mix readily, warm slightly and add a few drops of absolute alcohol.

4. Filter.

Loeffler's alkaline methylen blue.

Saturated alcoholic solution of methylen blue.....	30 c.c.
Potassium hydrate, 0.1% aqueous solution.....	100 c.c.

SOLUTIONS FOR USE IN STAINING

Aceton-alcohol for decolorizing in Gram's method of staining:

Aceton.....	10 c.c.
Absolute alcohol.....	100 c.c.

Acetic acid-alcohol for clearing in making impression preparations (also used for decolorizing in ordinary method of spore-staining):

Alcohol, 90%.....	2 parts
Acetic acid, 1%.....	1 part.

Mordant for staining flagella:

Tannin, 20%.....	10 c.c.
Ferrous sulphate, cold saturated solution..	8 c.c.
Fuchsin, cold saturated solution in absolute alcohol.....	1 c.c.

Lugol's iodine solution, for use in Gram's staining method:

Iodine.....	1 gm.
Potassium iodide.....	3 gms.
Distilled water.....	300 c.c.

STEAM TEMPERATURE PRESSURE TABLE

Temperature Centigrade.	Mm. of Hg.	Pounds per sq.in. Absolute Pressure.	Atmospheres.
<i>Degrees.</i>			
98	707.1	13.7	0.93
99	733.1	14.2	0.96
100	760.0	14.7	1.00
101	787.8	15.2	1.03
102	816.0	15.8	1.07
103	845.2	16.3	1.11
104	875.4	16.9	1.15
105	906.4	17.5	1.19
106	938.3	18.1	1.23
107	971.1	18.8	1.27
108	1004.9	19.4	1.32
109	1039.6	20.1	1.36
110	1075.3	20.8	1.41
111	1112.0	21.5	1.46
112	1149.8	22.2	1.51
113	1188.6	22.9	1.56
114	1228.4	23.7	1.61
115	1269.4	24.5	1.67
116	1311.4	25.3	1.72
117	1354.6	26.2	1.78
118	1399.0	27.0	1.84
119	1444.5	27.9	1.90
120	1491.2	28.8	1.96
121	1539.2	29.7	2.02
122	1588.4	30.7	2.09
123	1638.9	31.7	2.15
124	1690.7	32.7	2.22
125	1743.8	33.7	2.29

FORMULAE FOR CONVERSION OF DEGREES OF TEMPERATURE ON ONE SCALE INTO DEGREES ON ANOTHER

Centigrade (Celsius) scale: Freezing-point = 0°; boiling-point = 100°.

Fahrenheit scale: Freezing-point = 32°; boiling-point = 212°.

Réaumur: Freezing-point = 0°; boiling-point = 80°.

$$\text{Degrees C} \times 1.8 + 32 = \text{Degrees F.} \quad \text{Degrees } \frac{(F - 32)4}{9} = \text{Degrees R.}$$

$$\text{Degrees } \frac{F - 32}{1.8} = \text{Degrees C.} \quad \text{Degrees } \frac{R \times 5}{4} = \text{Degrees C.}$$

$$\text{Degrees } \frac{R \times 9}{4} + 32 = \text{Degrees F.} \quad \text{Degrees } \frac{C \times 4}{5} = \text{Degrees R.}$$

ALCOHOL BY VOLUME

TABLES

(From the Chemiker Kalender, published by Julius Springer, Berlin.)

Per Cent by Vol.	Specific Gravity.	Per Cent by Vol.	Specific Gravity.	Per Cent by Vol.	Specific Gravity.	Per Cent by Vol.	Specific Gravity.
1	0.9976	26	0.9689	51	0.9315	76	0.8739
2	0.9961	27	0.9679	52	0.9295	77	0.8712
3	0.9947	28	0.9668	53	0.9255	78	0.8685
4	0.9933	29	0.9657	54	0.9254	79	0.8658
5	0.9919	30	0.9646	55	0.9234	80	0.8631
6	0.9906	31	0.9634	56	0.9213	81	0.8603
7	0.9893	32	0.9622	57	0.9192	82	0.8575
8	0.9881	33	0.9609	58	0.9170	83	0.8547
9	0.9869	34	0.9596	59	0.9148	84	0.8518
10	0.9857	35	0.9583	60	0.9126	85	0.8488
11	0.9845	36	0.9570	61	0.9104	86	0.8458
12	0.9834	37	0.9559	62	0.9082	87	0.8428
13	0.9823	38	0.9541	63	0.9059	88	0.8397
14	0.9812	39	0.9526	64	0.9036	89	0.8365
15	0.9802	40	0.9510	65	0.9013	90	0.8332
16	0.9791	41	0.9494	66	0.8989	91	0.8299
17	0.9781	42	0.9478	67	0.8965	92	0.8265
18	0.9771	43	0.9461	68	0.8941	93	0.8230
19	0.9761	44	0.9444	69	0.8917	94	0.8194
20	0.9751	45	0.9427	70	0.8892	95	0.8157
21	0.9741	46	0.9409	71	0.8867	96	0.8118
22	0.9731	47	0.9391	72	0.8842	97	0.8077
23	0.9720	48	0.9373	73	0.8817	98	0.8034
24	0.9710	49	0.9354	74	0.8791	99	0.7988
25	0.9700	50	0.9335	75	0.8765	100	0.7939

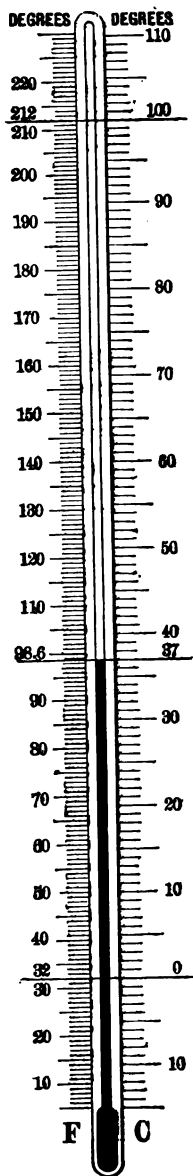


FIG. 74.—Comparison Fahrenheit-Centigrade Scale.

METRIC SYSTEM

Linear Measure

1000 millimicrons	= 1 micron (micromillimeter).
1000 microns	= 1 millimeter.
10 millimeters	= 1 centimeter.
10 centimeters	= 1 decimeter.
10 decimeters	= 1 meter.
10 meters	= 1 decameter.
10 decameters	= 1 hectometer.
10 hectometers	= 1 kilometer.
10 kilometers	= 1 myriameter.

The unit of length, one meter, is equal to $\frac{1}{1,000,000}$ part of the distance measured on a meridian of the earth from the equator to the pole and equals about 39.37 inches.

Square Measure

1,000,000 sq. millimicrons	= 1 sq. micron.
1,000,000 sq. microns	= 1 sq. millimeter.
100 sq. millimeters	= 1 sq. centimeter.
100 sq. centimeters	= 1 sq. decimeter.
100 sq. decimeters	= 1 sq. meter = 1 centare
100 sq. meters	= 1 sq. decameter = 1 are.
100 sq. decameters	= 1 sq. hectometer = 1 hectare.
100 sq. hectometers	= 1 sq. kilometer.
100 sq. kilometers	= 1 sq. myriameter.

Cubic Measure

1000 cubic millimeters	= 1 cubic centimeter.
1000 cubic centimeters	= 1 liter.
10 liters	= 1 decaliter.
100 liters	= 1 hectoliter.
1000 liters	= 1 kiloliter = 1 cu. meter = 1,000,000 c.c.

The unit of capacity is the liter and represents the volume of a kilogram of water at its maximum density, 4° C. and 760 mm. mercury pressure.

METRIC SYSTEM—*Continued*

Weight

The unit of weight is the gram and represents the weight of one cubic centimeter of water at its maximum density, 4° C. and 760 mm. mercury pressure.

10 milligrams	= 1 centigram.
10 centigrams	= 1 decigram.
10 decigrams	= 1 gram.
10 grams	= 1 decagram.
10 decagrams	= 1 hectogram.
10 hectograms	= 1 kilogram = 1000 grams.
10 kilograms	= 1 myriagram.
10 myriagrams	= 1 quintal.
10 quintals	= 1 millier or tonneau.

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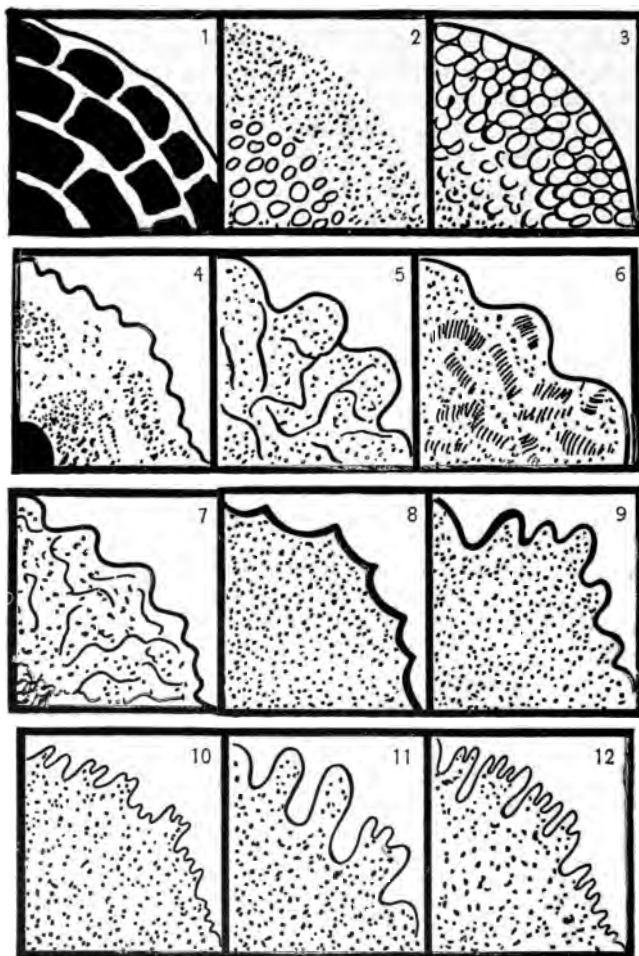
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- Journal of the Royal Army Medical Corps.
- Journal of Tropical Medicine.
- Lancet.
- Proceedings of the Royal Society of London.
- Revue générale du Lait.
- Zeitschrift für Hygiene und Infektionskrankheiten.
 - Zeitschrift für Immunitätsforschung.

A



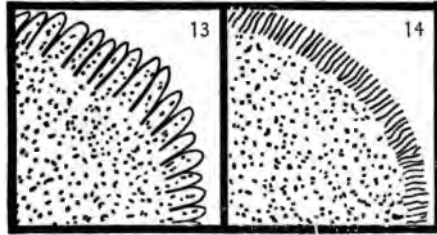
STRUCTURE OF COLONIES.

1. Areolate.
2. Grumose.
3. Moruloid.
4. Clouded.
5. Gyrose.
6. Marmorate.
7. Reticulate.

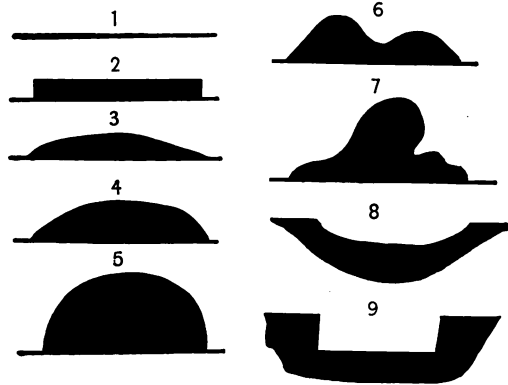
EDGES OF COLONIES.

8. Repand.
9. Lobate.
10. Eroze.
11. Auriculate.
12. Lacerate.
13. Fimbriate.*
14. Ciliate.*

* For illustration see next page.

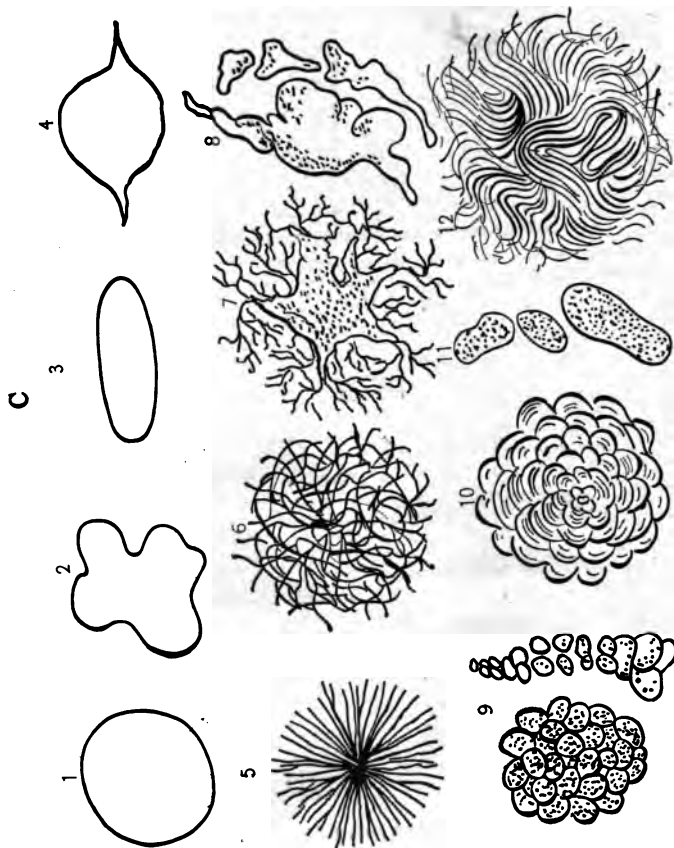


B



ELEVATION OF COLONIES.

1. Flat.
2. Raised.
3. Convex.
4. Pulvinate.
5. Hemispherical.
6. Umbilicate.
7. Umbonate.
8. Concave.
9. Rectangular depression.



FORM OF COLONIES.

1. Round.
2. Irregular.
3. Elliptical.
4. Spindle.
5. Myceloid.
6. Filamentous.
7. Rhizoid.
8. Cochleate.
9. Conglomerate.
10. Rosulate.
11. Toruloid.
12. Curled.

D*.

STAB CULTURES.

1. Filiform.
2. Beaded.
3. Papillate.
4. Villous.
5. Arborescent.

E*.

TYPES OF LIQUEFACTION.

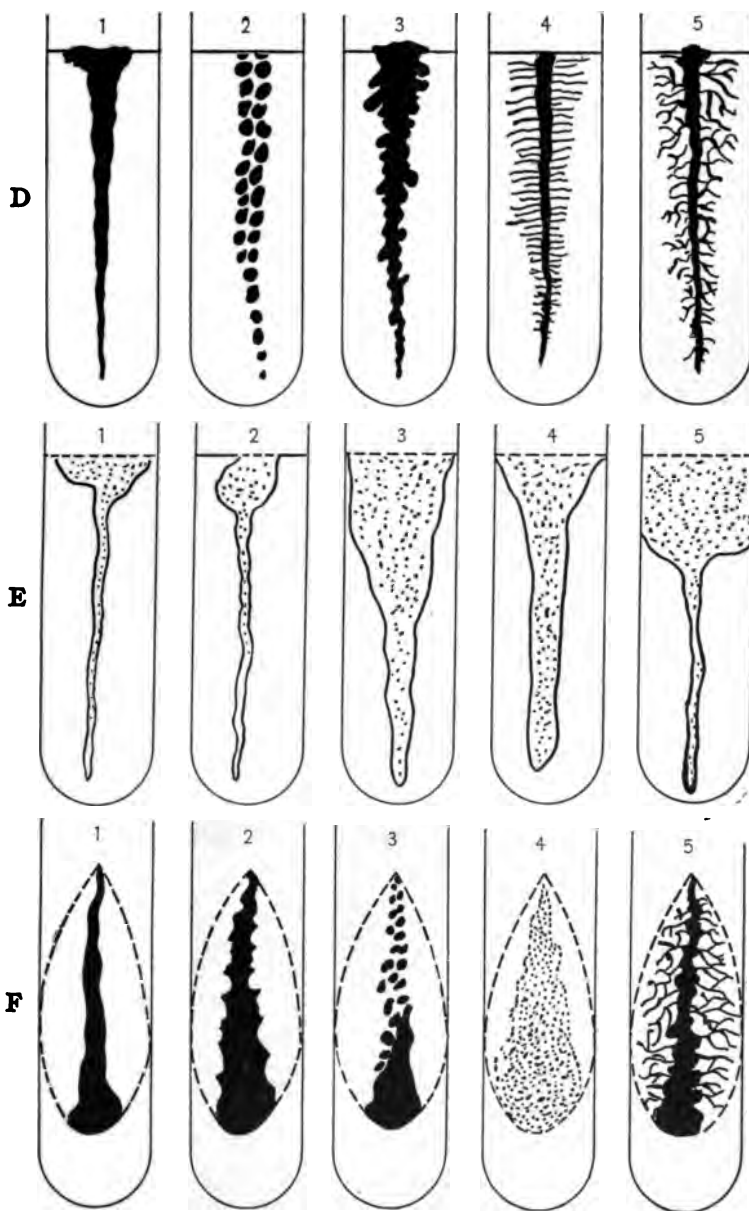
1. Crateriform.
2. Napiform.
3. Infundibuliform.
4. Saccate.
5. Stratiform.

F*.

STREAK CULTURES.

1. Filiform.
2. Echinulate.
3. Beaded.
4. Spreading.
5. Arborescent.

* For illustrations see next page.





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